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International Journal of Biological Technology (2010) 1(1):1-7.

Original Article

Investigation of Phytochemical constituents from *Eulophia epidendraea*

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Abstract

The tuber and leaf of *Eulophia epidendraea* was phytochemically examined. Chromatographic procedures led to the isolation of four phytochemicals in *β*-sitosterol (**I**), *β*-sitosterolglucoside (**II**), *β* – amyrin (**III**) and lupeol (**IV**) from the tuber and also four flavonoids of apigenin, luteolin, kaempferol, and quercetin were identified from the leaves of *E. epidendraea*.

Keywords: Orchidaceae, *Eulophia epidendraea*, tuber, leaf, Phytochemicals

Introduction

Higher plants are major sources of natural products such as pharmaceuticals, agrochemicals ingredients of flavor and fragrance, food additives, and pesticides (Balandrin and Klocke,1988). The pharmaceutically well known phychemical of morphine was isolated from opium poppy, *Papaver somniferum* by Sertuner (Burger,1960) quinine from *Chinchona officinalis* (Cragg *et al*.,2002), reserpine from *Rauvolfia serentina*, ephedrine from *Ephedra vulgaris* and taxol from *Taxus brevifolia* (Wani *et al*.,1971). These phytochemicals constitute some of the most exiting chemotherapeutic agents currently available for use in a clinical medicine.

Orchid *Eulophia epidendraea* (Retz.) Fischer belongs to the family Orchidaceae, Which has been traditionally used by the local people of Yadav community for the treatment of tumour, abscess and healing of wound (Maridass *et al.,* 2008). Recently, pharmacological studies on the wound - healing activity of tuber extract of *E. epidendraea* were reported. The present study was, therefore, carried out to identify the chemical constituents of the tuber and leaf of *Eulophia epidendraea* (Retz.) Fischer.

Materials and Methods

Plant materials

The orchid *Eulophia epidendraea* (Retz.) Fischer was collected from Kambli Malaikovil Forest, (75°50'E and 9°20'N) near Tenkasi, Tirunelveli District, Tamil Nadu, India.

Solvent extraction and isolation

The tuber of *Eulophia epidendraea* (Retz.) Fischer was air - dried and powdered. About 1.0 kg of this powder was extracted with petroleum ether (30-60°C) benzene (80.3°C), chloroform (61°C), acetone (56°C) and methanol (65°C) in a Soxhlet apparatus. The extraction process was performed for 8h. The solvents were evaporated under reduced pressure. After determining the yields, sediment extracts were stored at 4°C for further study. The methanolic extract (8.0g) was then fractionated by column chromatography on silica gel and eluted with ethyl acetate (EtOAc) followed by EtOAc - MeOH, and gradient 25 ml fractions were collected: fractions1- 4 (EtOAc), fractions 5-17 (10% MeOH), fractions18-21 (20% MeOH), fractons 22-33 (30% MeOH), fractions 34-38 (40% MeOH), fractions 39-56 [EtOAc-MeOH-H2O (10:5:1v/v/v)]. Fractions 5-17 contained compound (**I**). Fractions 18- 21 contained (**II**); fractions 34-38 contained (**III**); fractions 39-56 contained (**IV**) respectively. Identifications were made by comparison with the data from previous IR, UV, NMR and mass spectra (Pandey *et al*., 1996; Mučaji *et al*., 2000).

Instrumentation

UV spectra were obtained on a Shimadzu UV-160 spectrophotometer, and IR spectra were determined in KBr discs on a Perkin-Elmer 781 spectrophotometer. ^{1}H NMR spectra were recorded with a Varian Gemini NMR spectrometer at 200, 400 MHz or with a Bruker Avance NMR spectrometer at 500 MHz in $CDCl_3$. ^{13}C NMR spectra were recorded with a Varian Gemini NMR spectrometer at 50, 100 MHz or with a Bruker Avance NMR spectrometer at 125 MHz in $CDCl_3$. EI - MS were obtained with a JEOL JMS - HX110

spectrometer and HREI - MS with a Finnigan MAT 95S spectrometer.

Isolation of leaf flavonoids

The powdered leaf of *Eulophia epidendraea* was studied using hydrolyzed extract following the method of Harborne (1973). About 100g of dried leaves were cut into small pieces and extracted in 20 ml of 2M HCl, then boiled in a water bath at 100 ^{o}C for 1hr. The hydrolyzed extract was allowed to cool and filtered through a filter paper to remove debris from the extract. The filtrate was treated twice with ethyl acetate; the upper layer containing flavones and flavonol was then separated from lower aqueous layer by a separating funnel. Amyl alcohol was added to the latter layer to extract anthocynidins. These extracts were allowed to evaporate to dryness overnight in a dark fume chamber. Then five drops each of ethanol (95%) and methanol (100%) were added to dissolve flavones and flavonol which were ready for spotting into the plates.

Thin layer chromatograpic (TLC) analysis

The hydrolyzed extracts were run single dimensionally in solvent forestal, at room temperature of 21 - 28 ^{o}C. The concentrated extracts were spotted on the lower left corner of the TLC plate using 5µl micropipette. Fifteen loads of the extracts were applied and allowed to dry using a hair dryer before each subsequent load. The diameter of the spot in each chromatogram was normally about 5mm. Authentic markers of flavones (luteolin and apigenin) and flavonols (myricetin, quercetin and kaempferol) obtained commercially were co-chromatographed. Identification of the hydrolyzed compounds of these extracts was made by examination of the spots under UV light and by changes in colour under day light after application of ammonia. Rf values of these spots in comparison with the Rf values of authentic markers, coupled with those values given for each known compound in Harborne (1973), were of great help in identification of these spots. Nine chromatographic spots were identified in this study. They indicated four known compounds and five unidentified compounds (Table -2).

Results and Discussion

The methanolic-tuber extract of *Eulophia epidendraea* was subjected to a series of chromatographic separations over silica-gel, resulting in the isolation of *β*-sitosterol (**I**), *β*-sitosterolglucoside (**II**), *β* – amyrin (**III**) and lupeol (**IV**). The structures of these compounds were determined by comparing their spectral data with those reported or analyzing their various ^{13}C and ^{1}H- NMR spectral data and determined in comparison with the literature data. Compound (**I**) was obtained as colourless needle. The molecular formula $C_{29}H_{50}O$ was assigned by HREI-MS spectrophotometer. On the basis of ^{1}H and ^{13}C-NMR spectral data from the previous literature compound (**I**) was established as *β* – sitosterol (Kovganko *et al*., 1999).

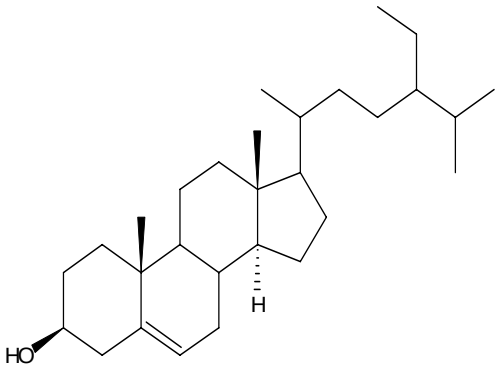

Fig.1. *β*- Sitosterol

In the present study, *β*-sitosterol was isolated from tuber of *Eulophia epidendraea*, and the same was reported in many species including *Tephrosia strigosa* and *Heliotropium indicum*, *Ajuga macrosperma* aerial part of *Brillantaisia palisatii, Elaphoglossum spathulatum, Parahancornia amapa, Conyza bonariensis, Lilium longiflorum* and *Tulipa gesneriana*, Zhongguo zhongyao, *Atractylodes chinenese* (Sreenivasulu and Sarma, 1996; Pandey *et al*., 1996; Dinda *et al*.,1997; Carvalhoa *et al*.,2001, Kong *et al*.,2001; Berrondo *et al*.,2003; Socolsky *et al*.,2003; Endoh *et al*.,1981). The occurrence of *β*-sitosterolglucoside was reported from plants such as *Lilium candidum, Olea europaea* and *Heliotropium indicum (*Pandey *et al*., 1996; Mučaji *et al*., 2000; Kadowaki *et al*., 2003). The intake of *β*-sitosterolglucoside capsule in marathon runner provide less inflammatory and reduced immunosuppressed activity excessive of physical stress (Bauic *et al*.,1999).

Compound (**II**) was obtained as colourless needle. The molecular formula $C_{35}H_{60}O_6$ was assigned by HREI-MS

spectrophotometer. On the basis of ^{1}H and ^{13}C-NMR spectral data from the previous literature compound (**II**) was established as *β* - sitosterolglucoside. (Swift, 1952). The ^{13}C-NMR spectrum of the *β* - sitosterol showed 35 carbon signal, including the signals corresponding to two olefinic carbon at δ121.9(C-6) and δ140.9 (C-5). Furthermore, the ^{1}H-NMR spectrum exhibited one olefinic proton signal at δ5.35 (H-6), two angular methyl groups at δ0.89(s,H-18), δ0.93(s,H-19) an isopropyl (δ0.86(H-26),0.89(H-27), 1.68 (H-25) and ethyl (δ0.66(H-29),1.26(H-28) group (Fig.1.)

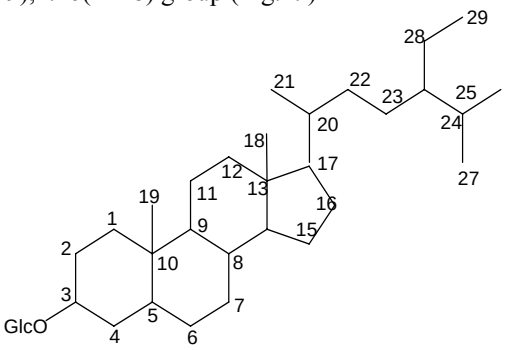

Fig.2. *β*- Sitosterolglucoside

Strong absorption due to many hydroxyl group (3400 cm^{-1}) in the IR spectrum and the signal in the ^{1}H-NMR (δ3.97 (H-5'),4.07(H-2'),4.30(H-3',4'), 4.43(H-6'α),4.58(H-6'β),5.06 (H-1') and ^{13}C-NMR (δ62.8 (C-6'), 71.7 (C-4'),75.4 (C-2'),78.6 (C-3'),102.6 (C-1') spectra suggested that the compound was a steroidal glycoside. This is the first report of steroidal glycoside from this orchid genus.

Beta-sitosterol (*β*-sitosterol) and its glucoside (*β*-sitosterolglucoside) are the most abundant sterols found in plants. In common with other phytosterols they are not endogenously synthesised in the human body and are derived exclusively from the diet (Ling *et al*.,1995). Although they differ from cholesterol by only an extra ethyl group in the side chain, they show profound biological effects in a number of experimental animal models. These include, inter alia, reduction of carcinogen-induced colon cancer, anti-inflammatory (Yamamoto *et al*., 1991), and anti-complement activity (Yamada *et al*., 1987). Bouic *et al*., (1996), reported that the *β*-sitosterol and *β* -sitosterol glucoside stimulate the proliferation of human peripheral blood lymphocytes and they can be used as an immunomodulatory agents. Donald *et al*., (1997) reported that the *β*-sitosterol and *β*-sitosterolglucoside were used in the treatment of pulmonary tuberculosis.

Compound (III)

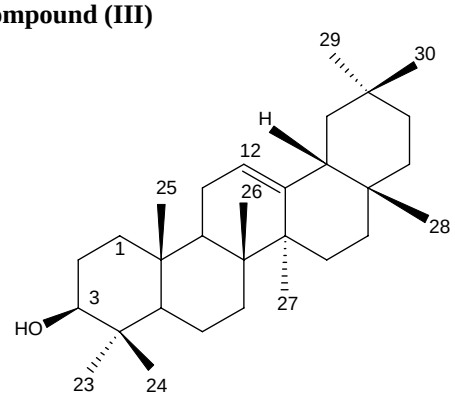

Fig.3. *β*- Amyrin

Compound *β*- Amyrin was obtained as colourless needle. The molecular formula was established as $C_{30}H_{50}O$ on the basis EI-MS: m/z 426 (M^{+}, 0.6%), 411(0.1%), 218 (100%), 272 (0.1%), 189 (30%), 135 (34%), 95(48%); UV spectrum showed the maxima at 240.2 nm (UV λ_{max}(MeOH); IR spectrum suggested the presence of IR γ_{max} (KBR) cm^{-1}: 3397, 2932, 1645, 1465, 1379, 1029, 900; ^{1}H (200MHZ, CDCl3): δ 5.12 (m, H-12), 3.23 (m, H-3), 1.13, 0.99, 0.97,0.94, 0.87(x2), 0.83, 0.79(CH_3). ^{13}C NMR (50.29 MHz,$CDCl_3$): δ 38.14 (C-1), 27.51(C-2), 79.12 (C-3), 38.88 (C-4), 55.27 (C-5), 18.44 (C-6), 33.03 (C-7), 38.82 (C-8), 47.81 (C-9), 37.00 (C-10), 23.48 (C-11),121.82 (C-12), 145.28 (C-13), 42.18 (C-14), 26.12 (C-15), 2737 (C-16), 32.04 (C-17), 47.22 (C-18), 46.93 (C-19), 31.34 (C-20), 34.83 (C-21), 37.26 (C-22), 28.22 (C-23), 15.47 (C-24), 15.72 (C-25), 16.97 (C-26), 25.26 (C-27),28.44 (C-28), 33.44 (C-29), 23.48 (C-30).

Compound (**IV**) was obtained as colourless needle. The molecular formula $C_{30}H_{60}O$ was assigned by HREI-MS spectrophotometer. On the basis of ^{1}H and ^{13}C-NMR spectral data from the previous literature compound (**IV**) was established as lupeol [Ref: Aratanechemuge *et al*., 2004].

In the present work, the compound *β*-amyrin was isolated from *Eulophia epidendraea* which was also found in several plants such as *Tephrosia strigosa* and *Heliotropium indicum*, *Brillantaisia palisatii*, *Lychnophora pinaster*,

Luxemburgia nobilis, *Chiococca braquiata, Parahancornia amapa, Atractylodes chinenese*, *Atractylodes chinenese* (Sreenivasulu and Sarma, 1996; Pandey *et al*., 1996, Carvalhoa *et al*.,2001; Ding *et al*.,2000; Berrondo *et al*.,2003, Silveira *et al*.,2005; Oliveira *et al*.,2002; Lopesa *et al*.,2004).

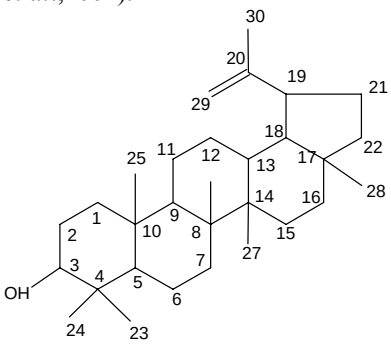

Fig.4. Lupeol

Similarly the compound Leupol isolated from *Eulophia epidendraea* was also reported from plants such as *Brillantaisia palisatii, Lychnophora pinaster, Parahancornia amapa,* (Ding *et al*.,2000; Carvalhoa *et al*.,2001; Berrondo *et al*.,2003, Silveira *et al*.,2005). Aratanechemuge *et al*.,(2004), reported that the suppression of growth of the HL-60 cells by lupeol results from the induction of apoptosis by this compound. Badami *et al*.,(2003) reported that the lupeol isolated from the bark of *Grewia tiliaefolia,* had weak cytotoxic properties.

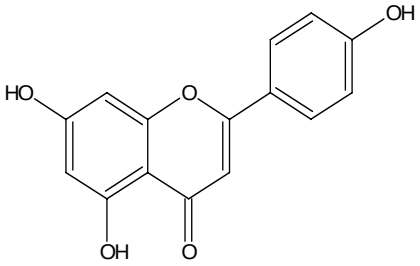

Fig.5. Apigenin

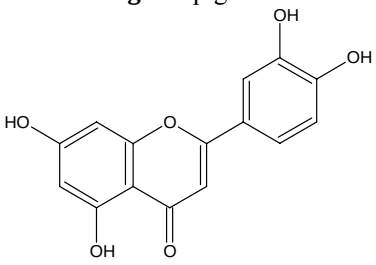

Fig.6. Luteolin

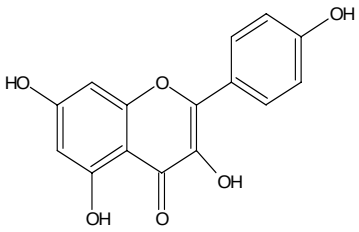

Fig.7. Kaempferol

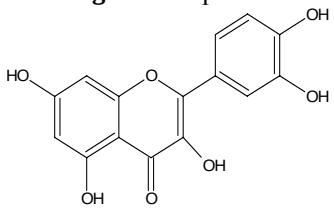

Fig. 8. Quercetin

Quercetin, a phytochemical belonging to the flavonoids, has antioxidant activities, inhibition of protein kinases (Davies *et al*., 2000) and DNA topoisomerases (Constantinou *et al*., 1995) regulate gene expression (Moon *et al*., 2003) and also modulate gene expression related to oxidative stress and in the antioxidant defence system (Moskaug *et al*.,2004). According to Van Wiel *et al*., (2001) and Tsanova- Savova and Ribarora (2002), the most common flavonoids in grape wine were flavonols (quercetin, kaempferol, and myricetin). Betes- Saura *et al*., (1996) detected quercetin, kaempferol in leaves and exocarps of grape *Vitis labruscana* cv. Kyoho and, *Vitis vinifera* L. fruits. *Lilium auratum, L. henryi, L.martagon, L. myrciphyllum* and *L. willmottiae. L. henryi, L. martagon, L. myrciphyllum, L. willmottiae, L. leichtlinii*.Apigenin and luteolin which were structurally elucidated from ^{13}C-NMR, where spectral data reported earlier (Loo *et al*., 1986; Wagner, 1976). These compounds were isolated from the roots of *Glossostemon bruguieri, Conyza bonariensis* (Meselhy, 2003; Kong *et al*., 2001). Luteolin was also isolated from the fruit of *Terminalia chebula (*Klika *et al*., 2004). So far anti-venom compounds isolated from plants include *β*-sitosterol, *β*-sitosterolglucoside, *β* - amyrin, kampferol and quercetin (Martz ,1992 ;Houghton *et al*.,1993; Abubakar *et al*.,2000; Reyes-Chilpa *et al*.,1994).t was concluded that the phytochemicals isolated from *Eulophia epidendraea* may appear to be a good resource of biologically active compounds.

Table- 1: ^{13}C- NMR ^{1}H- NMR data for lupeol (Aratanechemuge *et al*., 2004)

No.	^{13}NMR	^{1}H NMR
1.	38.7	1.65(1H,m),0.90(1H,m)
2.	27.4	1.59(1H,m),1.67(1H,m)
3.	79.0	3.20(1H,dd,J+5.03,11.5Hz)
4.	38.8	
5.	55.3	0.68(1H,m)
6.	18.3	1.40(1H,m),1.50(1H,m)
7.	34.3	1.32(1H,m),1.42(1H,m)
8.	40.8	
9.	50.4	1.29(1H,m)
10.	37.1	
11.	20.9	1.20(1H,m),1.40(1H,m)
12.	25.1	1.07(1H,m),1.68(1H,m)
13.	38.1	1.68(1H,m)
14.	42.8	
15.	27.4	1.00(1H,m)1.68(1H,m)
16.	35.6	1.37(1H,m) 1.48(1H,m)
17.	42.9	
18.	48.3	1.37(1H,m)
19.	47.9	2.38(1H,ddd,J=5.6,11.0,11.0Hz)
20.	150.9	
21.	29.8	1.37(1H,m),1.92(1H,m)
22.	39.9	1.37(1H,m),1.19(1H,m)
23.	27.9	0.97(3H,s)
24.	15.4	0.76(3H,s)
25.	16.1	0.83(3H,s)
26.	15.9	1.03(3H,s)
27.	14.5	0.94(3H,s)
28.	17.9	0.79(3H,s)
29.	109.3	4.54(1H,brs),4.67(1H,brs)
30.	19.3	1.68 (3H,s)

Table -2: Chromatoghapic identification of leaf phytochemicals

Spot No.	Mean Rf (x 100) in Forestal	Mean Rf (x 100) marker	Mean Rf (x 100) (Harborne ,1973)	Flavonoids	*Colour reactions*		
					Day light	UV-light	UV-ammonia
1.	-	79	83	Apigenin	Not visible	Ochre	Dull yellow
2.	22	29	-	Unknown1	Not visible	Light yellow	Bright yellow
3.	33	41	43	Unknown 2	Not visible	Light green	Olive green
4.	55	60	55	Kaempferol	Not visible	Yellow	Yellow
5.	66	60	66	Luteolin	Not visible	Ochre	Bright yellow
6.	38	44	41	Quercetin	Not visible	Yellow	Bright yellow
7.	72	78	-	Unknown3	Not visible	Yellow	Dark yellow
8.	68	-	-	Unknown 4	Not visible	Purple	Purple
9.	92	-	-	Unknown 5	Yellow	Bright yellow	Bright yellow

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References

Balandrin, M.J., and Klocke, J.A. 1988. Medicinal, aromatic and industrial materials from plants. (Editor Bajaj, Y.P.S.), Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plant, vol. 4. Springer-Verlag, Berlin, Heidelberg.

Burger, A. 1960. Medicinal chemistry. Inter Science publishers, Inc., New York, pp: 6-7.

Cragg, G.M., and Newman, D.J., 2002. Biodiversity: Medicinals for the new millennia. *Science and Culture*, 68(1-4): 3-10.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., and McPhail, A.T. 1971. Plant Antitumor Agents VI. The Isolation and Structure of Taxol, a Novel Antileukemic and Antitumor agent from *Taxus brevifolia*. *Journal of American Chemical Society,* 93: 2325-2327.

Pandey, D., Singh,J., Roy,R., Singh,V.B., and Pandey,V.P.1996. Constituents of *Heliotropium indicum*. *Orintal Journal of Chemistry*, 12 (3): 321-322.

Mučaji, P., Haladová, M., Eisenreichová, E., Buděšínský, M., and Ubik, K. 2000. Sterols in *Lilium candidum* L. (in Slovak). *Čes. Aslov. Farm.*, 49: 29-31.

Harborne, J.B. 1973. Phytochemical Methods. Chapman & Hall London.

Kovganko, N.V., Zh,N., Kashkan, E.V., Borisov, and Batura, E.V. 1999. ^{13}NMR spectra of β- sitosterol derivatives with oxidized rings A and B. Chemistry of natural compounds, 53(1), 646-649.

Aratanechemuge, Y., Hibasami, H.,Sanpin, H., Katsuzaki, H., Imai, K., and Komiya, 2004. Induction of apoptosis by lupeol isolated from mokumen (*Gossampinus malabarica* L. Merr) in human promyelotic leukemia HL-60 cells. *Oncology Reports,* 11: 289-292.

Ling, W. H., and Jones, P. J. H. 1995. Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sciences*, 57:195-206.

Yamamoto, M., Matsui, T., Sugiyama, K., Yakota, M., Nakagomi, K., and Nakazawa, H. 1991. Anti-inflammatory active constituents of *Aloe arborescens* Miller. *Agricultural and Biological Chemistry*, 55: 1627-1629.

Yamada, H., Yoshino, M., and Matsumoto, T. 1987. Effects of phytosterols on anti-complementary activity. *Chem Pharm Bull*.,35: 4851- 4855.

Bouic,P.J., Clark, A., Lamprecht, J., Freestone, M., Pool, E.J., Liebenberg, R.W., Kotze D., and Van Jaarsveld, P.P.1996. Beta-sitosterol and beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: implications for their use as an immunomodulatory vitamin combination. *International Journal of Immunopharmacology,* 18(12): 693-700.

Donald, P.R., Lamprecht, J.H., Freestone, M., Albrecht, C.F., Bouic, P.J.D., Kotze, D., and van Jaarsveld, P.P. 1997. A randomised placebo-controlled trial of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis. *International Journal of Tuberculosis Lungs Diseases*,1(5): 518-522.

Sreenivasulu, B., and Sarma, P.N. 1996. Chemical examination of the whole plants of *Tephrosia strigosa*. *Indian Journal of Chemistry* Sec., B, 1217-1218.

Carvalhoa, M.G., Vellosoa, C.R.X., Braz-Filhob, R., and Costa, W.F. 2001. Acyl-lupeol esters from *Parahancornia amapa* (Apocynaceae). *Journal of Brazilian Chemical Society,*12(4): 556-559.

Ding, H.Y., Wu, Y.C., and Ching, H. 2000. Phytochemical and Pharmacological Studies on Chinese Changzhu. *Journal of the Chinese Chemical Society*, 47: 561-566.

Berrondo, L.F., Gabriel, F.T., Fernandes, S.B.D.O., and Menezes, F.D.S. 2003. Dirhamnosyl flavonoid and other constituents from *Brillantaisia palisatii*. *Quimica Nova*, 26 (6): 922 - 923.

Silveira, D., De Souza Filho, J.D. De Oliveira, A. B., and Raslan, D.S., 2005. Lychnophoric acid from *Lychnophora pinaster*: a complete and unequivocal assignment by NMR spectroscopy. *Ecl. Quím., São Paulo*, 30(1): 37- 41.

Oliveira, M.C.C., Carvalhoa, M.G., Silva, C.J., and Werle, A.A. 2002. New biflavonoid and other constituents from *Luxemburgia nobilis* (EICHL). *Journal of Brazilan Chemical Society,* 13(1): 119-123.

Lopesa, M.N., Oliveira, A.D., Young, M.C.M., and Bolzani, V.D.S. 2004. Flavonoids from *Chiococca braquiata* (Rubiaceae). *Journal of Brazilian Chemical Society,* 15, (4): 468 - 471.

Badami, P., Vijayan, P., Mathew, N., Chandrashekhar, R., Godavarthi, R., Dhanaraj,

S.A., and Suresh, B. 2003. *In- vitro* cytotoxic properties of *Grewia tiliaefolia* bark and Lupeol. *Indian Journal of Pharmacology*, 35: 250-251.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemistry Journal*, 351: 95 - 105.

Moon, S.K., Cho, G.O., Jung, S.Y., Gal, S.W., Kwon, T.K., Lee, Y.C., Madamanchi, N.R., and Kim, C.H. 2003. Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cellcycle regulation, and matrix metalloproteinase-9. *Biochem. Biophys. Res. Commun*., 301: 1069-1078.

Moskaug, J. Ø., Carlsen, H., Myhrstad, M., Blomhoff, R. 2004. Molecular imaging of the biological effects of Quercetin and quercetin-rich foods. *Mechanisms of Ageing and Development*, 125:315–324.

Van Wiel, A., van Golde, P.H.M., and Hart HCh, 2001. Blessings of the grape. *European Journal of International Medicine,* 12: 484 - 489.

Betes-Saura, C., Andres - Lacueva, C., and Lamuela-Raventos, R. M. 1996. Phenolics in white free run juices and wines from penedes by high-performance liquid chromatography: changes during vinification. *Journal of Agricultural Food Chemistry*, 44: 3040-3046.

Loo, P. V., De Bruyn, A., and Budessinsky, M. 1986. Reinvestigation of the structural assignment of signals in the ^{1}H and ^{13}C NMR spectra of the flavone agigenin, *Magnetic Resonance in Chemistry*, 24: 879 - 882.

Wagner H., Chari, V. M., and Sonnenbichler, J. 1976. ^{13}C-NMR-spektren natürlich vorkommender flavonoide. *Tetrahedron Letter*, 1799-1802.

Meselhy R., and Meselhy, M.R. 2003. Constituents from *Moghat*, the roots of *Glossostemon bruguieri*(Desf.). *Molecules*, 8: 614-621, http://www.mdpi.org.

Kong, J.M., Khang, N.G., Sai1, C.L, and Fatt, C, T. 2003. Recent advances in traditional plant drugs and orchids. *Acta Pharmacology Sinca*, 24(1): 7-21.

Klika, K.D., Saleem, A., Sinkkonen, J., Kähkönen, M., Loponen, J., Tähtinen, P., and Pihlaja, K. 2004. The structural and conformational analyses and antioxidant activities of chebulinic acid and its thrice-hydrolyzed derivative, 2, 4-chebuloyl-*β*-D-glucopyranoside, isolated from the fruit of *Terminalia chebula. ARKIVOC*, (vii) 83-105.

chemotheraphy, 51: 241-246.

Martz, W., 1992. Plants with a reputation against snakebite. *Toxicon,* 30: 1131-1142.

Houghton, P.J., and Osibogun, I.M. 1993. Flowering plants used against snakebite. *Journal of Ethnopharmacology,* 39: 1-29.

Abubakar, M.S., Sule, M.I., Pateh, U.U., Abdurahman, E.M., Haruna, A.K., Jahun, B.M. 2000. *In - vitro* snake venom detoxifying action of the leaf extract of *Guiera senegalensis*. *Journal of Ethnopharmacology,* 69: 253-257.

Reyes-Chilpa, R., Gómez-Garibay, F., Quijano, L., Magos-Guerrero, G., and Ríos, T. 1994. Preliminary results on the protective effect of (-) - edunol, a pterocarpan from *Brongniartia podalyrioides* (Leguminosae), against *Bothrops atrox* venom in mice. *Journal of Ethnopharmacology,* 42: 199-203.

Swift, L.J. 1952. Isolation of *β*-sitosteryl- D-glucoside from the juice of Florida Valencia oranges (*Citrus sinensis*, L.). *Journal of American Chemical Society*, 74:1099-1100.

Maridass, M., Thangavel, K. and Raju, G.2008. Antidiabetic Activity of tuber extract of *Eulophia epidendraea* (Retz.) Fisher (Orchidaceae) in alloxan diabetic rats. *Pharmacologyonline,* 3: 606-617.

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Original Article

Pharmacognostical and phytochemical investigation studies on *Gymnema sylvestre* R.Br.

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Abstract

Gymnema sylvestre R.Br. is commonly known as "Gur-mar" in India and Vietnam well known for masking sweet taste. It is widely used in indigenous system of medicine for treatment of Diabetes mellitus. The aim of the present study was focused on the pharmacognostical, physico-chemical and phytochemical properties were carried out, which would like to facilitate quick identification and selection of the drug from various adulterates.
Keywords**:** *Gymnema sylvestre*; Asclepiadaceae; pharmacognostical, indigenous system, physico-chemical

Introduction

The use of plants as medicine is as old as human civilization. Peoples of all ages in both developing and undeveloped countries use plants in an attempt to cure various diseases and to get relief from physical sufferings. Herbal drugs, in India are also used as household remedy for common ailments since time immemorial. Our ancestors have a profound knowledge of these medicinal plants and they knew innumerable remedies, a fact indicated in the writings of *Siddhars* of Tamil Nadu. Their expertise if documented properly would help the modern man find more effective prophylactic use of these herbs. The relevance of pharmacognosy in standardization of herbal drugs was been long been stressed. Many monographs on pharmacognostic have emerged as an aid in the pharmacognostic investigations (Kalidass *et al*., 2009a;Edward,1956). The process of standardization can be achieved by stepwise pharmacognostic studies. These studies help in identification and authentication of the plant material (Ozarkar, 2005).

Gymnema sylvestre R.Br. is one of the important anti-diabetic medicinal plant, there is a growing demand for *G. sylvestre* leaves in the pharmaceutical trade. Gymnemic acid, the active indigradients of this plant, is extracted from leaves and used widely as anti-diabetic (Shanmugasundaram *et al*.,1983), anti-sweetner (Kurihara,1992) and antihypercholesterolemic (Bishayee and Chatterjee,1994). It also has stomatic, diuretic and cough suppressant property (Kapoor,1990 and Sastri,1956). The plant has been reported to possess antimicrobial (Sative *et al*.,2003) and ethno-veterinary medicinal properties (Kalidass *et al*.,2009b). Hence, because of these properties, *Gymnema sylvestre* is most important for plant prospecting. The present investigation an attempted has been made to evaluate various pharmacognostic standards like ash and extractive values, fluorescence analysis of aerial parts of the plant and preliminary phytochemical analysis of *Gymnema sylvestre*.

Materials and Methods

The plants of *Gymnema sylvestre* R.Br. (Asclepiadaceae) were collected from the well grown healthy plants inhabiting the natural forests of Kalakad Mundathurai Tiger Reserve Forest, Western Ghats, Tamil Nadu, India. The plant material was properly identified and confirmed with help of various floras (Gamble, 1991; Matthew,1991). *G. sylvestre*, a perennial plant, stout woody climber with long slender branches is distributed throughout India, in dry forests. The leaves of this plant are opposite, entire, 1 to 3 inches long, and 1 to 2 inches broad, elliptic or obovate, acute or cuspidate, rarely cordate at the base, membranous, thinly pubescent on both sides, the upper surface often darker green than the lower.

Physicochemical constant and fluorescence analysis

These studies were carried out as per the standard procedures (Lala,1993). In the present study, the powder of aerial part was treated with 1N aqueous sodium hydroxide and

1N alcoholic sodium hydroxide, acids like 1N hydrochloric acid, 50% sulphuric acid, nitric acid, picric acid, acetic acid and nitric acid with ammonia. These extracts were subjected to fluorescence analysis in visible/daylight and UV light (254nm & 365nm). Various ash types and extractive values were determined by following standard method by African Pharmacopoeia (1986) and Anonymous (1996).

Preliminary phytochemical analysis

Shaded dried and powdered of aerial part of plant samples were successively extracted with hexane, chloroform, ethanol and water. The extracts were filtered and concentrated using vaccum distillation. The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per standard procedure (Lala,1993; Brindha *et al.*,1981).

Results and Discussion

The detailed and systematic pharmacognostical evaluation would give valuable information for the future studies. In the present study, physical constant as ash value of the drug gives an idea of the inorganic composition and other impurities present along with the obtain from this plant species. Extractive values are useful for the determination of adulterated drugs. The results of the physical constants of the drug powder are given in table-1. For determining ash, the powdered drug is in aerated so as to burn out all organic matter. The ash value was determined by four different methods, which measured total ash, water soluble ash, and acid soluble ash. The total ash for aerial parts was found to be 8.22% of which, acid insoluble ash was 1.08% and water soluble ash was 3.39%. The extraction values were found to be 18.21% and 20.19% for water and alcohol respectively.

The extracts obtained by exhausting plant materials with specific solvents are indicative of approximate measure of their solvents from a specific amount of air dried plant material. The values depend on the chemical nature, quality, properties of constituents, the solvent employed, and the type of plant part and the method of extraction employed. This parameter is employed for materials for which a jet no suitable biological assay exists (Evans, 2002).

Table -1: Ash values and extractive values of the powdered aerial part of *Gymnema sylvestre*

S. No.	**Nature of the extract**	**Extractive value (%)**
1	Alcohol (Ethanol)	20.19 ± 0.01
2	Water (Aqueous)	18.21 ± 0.11
S. No.	**Type of Ash**	**% of Ash**
1	Total ash value of powder	8.22 ± 0.15
2	Water soluble ash	3.39 ± 0.04
3	Acid insoluble ash	1.08 ± 0.01

The results showed greater extractive values in hot extraction indicating the effective of elevated temperature on extraction. In all methods alcohol has unique feature of dissolving all polar and nearly all new polar constituents (Mukherjee,2002). Studies on physico-chemical constants can be serving as a valuable source of information and provide suitable standards to determine the quality of this plant. Many phytochemical fluoresce when suitably illuminated. The fluorescence colour is specific for each compound. A non-fluorescent compound may fluoresce if mixed with impurities that are fluorescent. The fluorescent method is adequately sensitive and enables the precise and accurate determination of the analyze over a satisfactory concentration range without several time consuming dilution steps prior to analysis of pharmaceutical samples (Pimenta *et al.*, 2006). In the present study is the powdered aerial parts of *G. sylvestre* emitted light green under short UV light and dark green in long UV light (Table 2).

Table -2: Fluorescence analysis of the powdered aerial part of *G. sylvestre*

Experiments	Visible/Day light	UV Light	
		254nm	365nm
Drug powder as such	Green	Green	Light green
Powder + 1N NaOH (aqueous)	Brown yellow	Light green	Dark green
Powder + 1N NaOH (alcohol)	Light yellow	Fluorescent green	Orange
Powder + 1N HCL	Brown	Light brown	Brown
Powder + 50% H_2So_4	Brown	Light blue	Light green
Drug powder + Nitric acid	Reddish brown	Light green	Light green
Drug Powder + Picric acid	Green	Fluorescent green	Green
Drug Powder + Acetic acid	Yellow	Fluorescent green	Fluorescent green
Drug Powder + Ferric chloride	Light brown	Light green	Green
Drug Powder + HNO_3 + NH_3	Light brown	Light green	Pale green

Table -3: Phytochemical screening of the powdered aerial part of *G. sylvestre*

No.	Test	Hexane	Chloroform	Ethanol	Water
1	Alkaloids	-	+	+	+
2	Terpenoids	+	+	-	-
3	Steroids	-	+	-	-
4	Coumarin	+	-	-	-
5	Tannin	+	-	-	+
6	Saponin	-	-	-	+
7	Flavonoids	-	-	+	-
8	Quinones	-	+	-	-
9	Anthraquinones	-	-	+	-
10	Phenol	+	+	+	+
11	Xanthoprotein	-	-	+	-
12	Carbohydrate	-	-	+	+
13	Glycosides	-	-	+	-
14	Fixed oil	+	+	-	-

The results of preliminary phytochemical screening of aerial part of plant extracts of *G. sylvestre* are presented in table 3. The ethanol extracts of the leaf shows the presence of alkaloids, terpenoids coumarin, tannin, saponin, flavonoids, phenols, anthraquinones, quinones, carbohydrate and glycosides. This is comparable with values reported for several medicinal plants such as *Gynandropsis gynandra* and *Buchholzia coriaceae* (Ajaiyeoba,2000); *Erythrina senegalensis* (Bako and Madu, 2007); *Vitex negundo* (Panda *et al*., 2009); *Terminalia glaucescens* (Adebayo and Ishola, 2009). The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences. For instance saponin is used as mild detergents and in intracellular histochemistal staining. It is also used to allow antibody access in intracellular proteins. In medicine, it is used in hypercholesterolaemia, hyperglycaemia, antioxidant, anticancer, anti-inflammatory, etc. it is also known to have antifungal properties (De-Lucca *et al*.,2005).

Pharmacognostic studies and phytochemical screening can serve as a basis for proper identification, collection and investigation of the plant. These parameters are to be useful in the preparation of the herbal monograph for its proper identification. Any crude drug which is claimed to be *G. sylvestre*, but whose characters significantly deviate from the above accepted standards would then be rejected as contaminated, adulterated or downright fake. Since *Gymnema sylvestre* is used in various medicinal preparations; Hence the present study may be useful to supplement information in respect to its identification, authentication and standardization.

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References

Adebayo, E.A., and Ishola, O.R. 2009. Phytochemical and antimicrobial screening of crude extracts of *Terminalia gaucescens*. *Afri. J. Pharm. Pharmacol*.,3(5): 217 - 221.

African Pharmacopoeia, 1986. General Methods for Analysis 1st Ed. 2: (OAU/STRC) *Lagos*. pp. 123.

Ajaiyeoba, E.O., 2000. Phytochemical and antimicrobial studies of *Gynandropsis gynandra* and *Buchholzia coriaceae* Extracts. *Afr. J.Biomed. Res*., 3(3): 161 – 165.

Anonymous, 1996. Indian Pharmacopoeia, Vol. I & II. Government of India, Ministry of Health and Family Welfare, the Controller of Publications, Civil Lines, Delhi – 110054, 1996.

Bako, S.S. and Madu, P.C. 2007. Phytochemical and antimicrobial investigation of crude extracts of the leaves of *Erythrina senegalensis*. *Ind. J. bot. res*. 3(1): 17 - 22.

Bishayee, A. and Chatterjee, M., 1994. Hypolipidaemic and antiatherosclerotic effects of oral *Gymnema sylvestre* R. Br. Leaf extract in albino rats fed on a high fat diet. *Phytothera. Res.*, 1994, 8, 118-120.

Brindha, P., Sasikala, P. and Purushothaman, K.K. 1981. Pharmacognostic studies on *Merugan kizhangu*. *Bulletin of Medico Ethnobotanical Research*, 3: 84-96.

De-Lucca, A., Cleveland, T., Rajasekara, K., Bouc, S. and Brown, R. 2005. Fungal properties of CAY-1 a plant saponin, for emerging fungal pathogens. 45th interscience conference in antimicrobial agents and chemotherapy abstract, F-490, 180.

Edward, P.C.1956. Leaf and Febiger Philadelphia,731.

Evans, W.C. 2002. Trease and Evans pharmacognosy, (W.B. Saunders, Edinburgh, 2002): 540 - 541.

Gamble J.S. 1991. Flora of the Presidency of Madras. Reprinted Edition, Vol I – III, Bishen singh Mahenra Pal Singh, Dehra Dun.

Kalidass, C., Shanmugasundaram, R. and Mohan, V.R. 2009a. Pharmacognostic studies of *Capparis separia* (L.) R.Br. *Pharmacognosy Journal*. 1(2): 121 - 125.

Kalidass, C., Muthukumar, K., Mohan, V.R. and Manickam,V.S.2009b.Ethnoveterinary medicinal uses of plants from Agasthiamalai Biosphere Reserve (KMTR), Tirunelveli Tamil Nadu, India. *My Forest*, 45(1): 7 – 14.

Kapoor, L.D. 1990. *CRC* Handbook of Ayurvedic medicinal plants: Boca Raton, Fl. pp: 200 - 201.

Kurihara, Y. 1992. Characteristics of anti-sweet substances, sweet proteins and sweetness inducing protein. *Crit. Rev. Food Sci. Nutr.*, 32: 231-252.

Lala, P.K.1993. Lab Manuals of Pharmacognosy, CSI Publishers and Distributors, 5 th Edition, Culcutta.

Matthew, K.M. 1991. An Excursion Flora of Central Tamil Nadu, India. Oxford and IBH Publishing Co., New Delhi.

Mukherjee, P.K. 2002. Quality control of herbal drugs: An approach to evaluation of botanicals) (Business Horizons, New Delhi, 2002). pp.362.

Ozarkar, K.R. 2005. Studies on anti-inflammatory effects of two herbs *Cissus quadrangularis* L. and *Valeriana wallichi* DC. Using mouse model, *Ph.D*. thesis, University of Mumbai, Mumbai.

Panda, S.K., Thatoi, H.N., and Dutta, S.K. 2009. Antimicrobial activity and phytochemical screening of leaf and bark extracts of *Vitex negundo*. *J. Med. Plant. Res*. ,3(4): 294 - 300.

Pimenta, A.M., Montenegro, M.C., Araujo, A.N. and Martine, J.C. 2006. Application of sequential injection analysis to pharmaceutical analysis. *Journal of Phar. Biomed. Annuls*. 40: 16 - 34.

Sative, R.K., Abhilash, P. and Fulzele, D.D.2003. Antimicrobial activity of *Gymnema sylvestre* leaf extract. *Fitoterapia*. 74: 699- 701.

Shanmugasundaram, K.R, Panneerselvam, C., Samudram, P. and Shanmugasundaram, E.R. 1983. Enzyme changes and glucose utilisation in diabetic rabbits: the effect of *Gymnema sylvestre*, R.Br. *J. Ethnopharmacol*.,7:205-234.

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Original Article

Phytochemical, Pharmacognostical, Antimicrobial activity of *Indigofera aspalathoids* vahl. (Fabaceae)

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Abstract

Our present study is aimed to detect the medicinal uses of the plant Indigofera *aspalathoides* Vahl. belonging to the family Fabaceae by performing various studies such as Phytochemical, Pharmacognostical and Antibacterial activity, using seven different bacterial strains, which are harmful to human beings. The *Indigofera aspalathiods* commonly known as 'Sivanar vembu' has been recognized in different system of traditional medicines for the treatment of different diseases and ailments of human beings. The leaves, flowers and tender shoots of the plant are said to be cooling and demulcent and are employed as decoction in leprosy and cancerous infections. The root is chewed as remedy for toothache. The whole plant rubbed up with butter is applied to reduce oedematous tumour. A preparation made from the ashes of the burnt plant is used to remove dandruff from the hair and oil for syphilitic and other skin diseases. A decoction of the entire plant is given as an alternative in secondary syphilis and psoriasis.

***Keywords*:** *Indigofera aspalathoids*, Sivanar vembu, Phytochemical, Pharmacognostical, Antibacterial activities

Introduction

Plants contain chemical compounds that may be in one way or another responsible for their healing properties and other functions. The chemical compounds are secondary metabolites of which at least twelve thousand have been isolated (Hasan *et al.*, 1988).

Phytochemistry deals with the analysis of plant chemicals called natural products, and with changes occurring in such chemicals due to alterations in environmental conditions. These compounds are involved as well in allelopathy, dealing with the interactions between two plants, which process can change depending upon variations in the phytochemicals produced under particular environmental conditions (Zobel *et al*., 1999).

Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subjects for very intense pharmacological studies; this has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutic value and as sources of lead compounds in the drug development. In developing countries, it is estimated that about 80% of the population rely on traditional medicine for their primary health care. There arises a need and therefore to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies (Shailendra Gurav *et al.*, 2007). Up to 80% of the population depends directly on the traditional medicine for the primary health care (Kirby, 1996).

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are chiefly due to synthesized during secondary metabolism of the plant (Prusti, 2008).

The present study is aimed to investigate the phytochemical, pharmacognostical and antibacterial activity of the medicinal plant *Indigofera aspalathoids* Vahl. which belongs to the family Fabaceae.

Materials and Methods

For the present study *Indigofera aspalathoids* Vahl. belongs to the family Fabaceae was subjected to study the phytochemical, pharmacognostic and antimicrobial bacterial activity. The plant was collected from Sivanthipatti hills in Tirunelveli District of Tamil Nadu during the month of January 2009.

Macroscopic (Taxonomic) Studies

The plant was collected during the month of January 2009 and well preserved. The observation was made by using student dissection microscope and the details were described in technical terms.

Microscopic (Anatomical) Studies

Fresh plant of *Indigofera aspalathoids* Vahl. (Fabaceae) was collected and fixed in FAA (Formalin Acetic acid and alcohol mixtures). Free hand section of stem, leaf and root were taken and kept in 70% ethanol. The sections were stained with saffranin and mounted according to the methods described by Johansen (1940). The photomicrographs were taken using Motic digital camera, and Phase Contrast Microscope, Japan.

Phytochemical Studies

Mature and healthy plants were collected and dried at room temperature (25–30˚C) for about two weeks. The dried plants were ground to powder. 5 grams of the powdered plant was put in to a bottle and shaken with a mechanical shaker for 12 hours. Then filtered with Whatman No. 1 filter paper to obtain petroleum ether, benzene, chloroform, ethanol and distilled water extracts. The qualitative phytochemical analysis was carried out on the extracts to determine the presence or absence of reducing sugar, protein, phenolic groups, alkaloids, steroid, triterpene, flavone, catachin, tannin and anthraquinone (Trease and Evans, 1996).

Pharmacognostic Studies

1. ***Fluorescence Analysis***

Fine powder and their extracts were obtained in various solvents namely petroleum ether, benzene, chloroform and ethanol. The aqueous extract was prepared by directly boiling the powder with distilled H_2O. They were examined under visible and U.V light. These powdered material were also treated with various reagents such as 50% $HN0_3$, acetone, ethyl alcohol, 50% H_2S0_4, 1N HCl and 1N aqueous NaOH and changes in colour were recorded.

2. ***Quantitative Determination***

The percentage of total ash, water soluble ash, acid insoluble ash, sulphated ash, were obtained by employing standard methods of analysis as described in Pharmacopoeia of India (1996).

3. ***Qualitative Determination***

Tests for detection of inorganic elements in plant ash.

Preparation of Ash Extracts

For detection of various elements in plant ash Ca, Fe, S, P and Cl, one gram ash material was dissolved in 25ml of 50% HCl for 12 hours and then filtered through filter paper. The filtrate was treated with suitable reagents to identify the presence of elements qualitatively.

Antibacterial Activity

A. ***Materials***

a. Microbial Strains

b. The different bacterial strains used for the study of antibacterial activities were collected from the Vivek laboratories, K.P.Road, Nagercoil and maintained by sub culturing. The bacteria's were sub-cultured in the Mullen-Hinton agar medium. The samples for bacterial strain were sub cultured in individual plates.

Solvant

The organic solvents such as petroleum ether, benzene, chloroform, ethanol and distilled water were used to extract bioactive compounds.

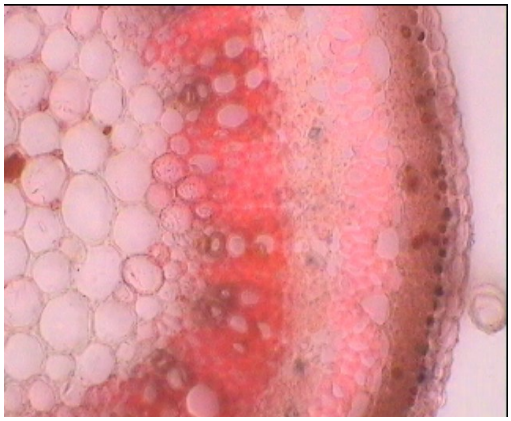
Methods

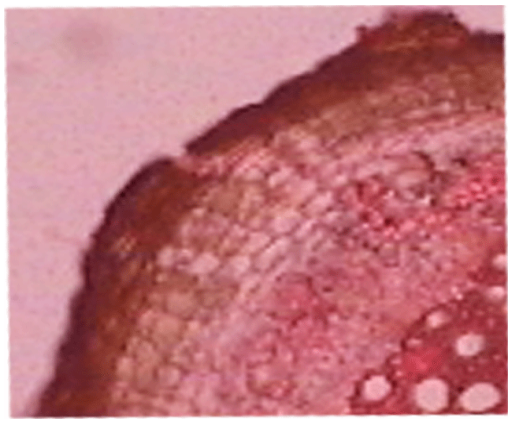
1. Extraction of antibacterial compounds
2. Maintenance of microbial strains
3. Nutrient media and nutrient broth
4. Preparation of sterile antibiotic discs
5. Plating and Assay of antimicrobial activity

Results and Discussion

The plant is an erect much branched stiff under shrub and grows erect. Stem is dark brown, when young, grayish white, branched 0.7cm to 1.5cm width. Young branches silvery pubescent and are characteristically purple with dense covering of minute trichomes; Roots are brown colored, woody, lateral roots present 0.5 to 2.0cm width. No characteristic odour. The

leaf is 1-3 trifoliate, pale green, oblanceolate, digitate, sessile and crowded on the young branches, stipules minute. Leaflets usually 3, thick, oblanceolate, folded; The flowers are purple, solitary and auxillary, pedicels slender, 2 cm in long; The pods are straight and cylindrical with sparse trichomes usually 6 cm in long. Seeds are cubical and smooth. The microscopic structures of stem and root was shown in photos.





The results of the phytochemical analysis shows that except napthoquinone all other secondary metabolites such as steroids, triterpenes, alkaloids, phenolic groups, flavone groups, saponin, tannin, sugar, catachin, aminoacids and reducing sugar are present in the plant.

Fluorescence analysis of the plant powder in various solvents have been studied and presented in table. It can be as a diagnostic tool for testing adulterations, if any. Under fluorescent light the plant powder showed different colors in various extracts.

The total ash content of *Indigofera aspalathoids*. is 18.06%. The acid insoluble ash content of *Indigofera aspalathoids* Vahl. Is 1.13%. The sulphated ash content of *Indigofera aspalathoids* Vahl. is 17.89%. The water soluble ash content in *Indigofera aspalathoids* Vahl. is 1.24%. Thus, the macroscopic characters, preliminary phytochemical screening and pharmacognostic studies can be used as a diagnostic tool in the correct identification of the plants and also to identify adulteration of these materials.

The plant *Indigofera aspalathoids* Vahl. shows the presence of almost all minerals such as sulphur, phosphorous, irons and calcium except chlorine.

Antibacterial activity of the extract of *Indigofera aspalathoids* Vahl. in different solvent extracts are shown in table.

The antibiotic disc ampicilin showed antibacterial activity against *Stapphylococcus aureus*, *Staphylococcus epidermidis*, *E.coli*, *Klebsiella* sps. and *Enterobacter* sps. It doesn't show any activity against *Proteus vulgaris* and Proteus mirabilis. It had the maximum inhibitory action against almost all the bacteria used for example *Streptococcus aereus* 14mm. Therefore it should be understand that by undergoing these studies various secondary metabolites present in the plant, its importance for used as drug, its activity to fight against certain bacteria which are harmful to human beings can be easily identified. Further integrated investigation using HPLC and GC - MS will lead to purification and structural elucidation of active principles against microorganisms.

Table-1: Flourescence analysis of the extracts of *indigofera aspalathoids* Vahl.

Sl.No	Treatment	Under visible Light	Under UV Light
1	Powder as such	Yellowish green	Light green
2	Petroleum ether extract	Light yellow	Light green
3	Chloroform extract	Yellowish green	Green
4	Ethanol extract	Light green	Dark green
5	Distilled water extract	Light orange	Green
6	Powder +1N aqueous NaOH	Brown	Green
7	Powder +acetone	Pale yellow	Light green
8	Powder +N HCl	Orange	Green
9	Powder +50%HNO_3	Reddish orange	Dark green
10	Powder +50%$H_2 SO_4$	Yellowish brown	Dark green

Table- 2: Antibacterial activity of *Indigofera aspalathoids*

Tested organism	Benzene	Ethanol	Petroleum ether	Chloroform	Control
S. aureus	11	11	11	25	25
S. epidermis	11	-	-	25	24
Escherichia eoli	-	-	-	25	27
Klebsiella sp	-	-	-	17	12
Proteus vulgaris	25	-	23	23	-
Proteus mirabilis	25	-	22	24	-
Enterobacter sp	11	10	-	13	24

References

Hasan C.M., Islam S.N., Begun K., Ilias M. and Hassain A. 1988. Antibacterial activities of the leaves and stem barks of *Cassia alata*. *Bengladesh J. Bet*.17:135-139.

Johanson, 1940. Plant Microtechnique Stains Mc GRAW – *HILL Publications*. 40 – 50.

Kirby,G.C.1996. Medicinal plants and the control of parasites. *Trans. Roy. Soc. Trop. Med. Hyg.* 90:605-609.

Prusti,A., Mishra, S.R., Sahoo, S. and Mishra S.K.2008. Antibacterial Activity of Some Indian Medicinal Plants Department of Botany, P.N. College, Khurda-752057. *Orissa University Department of Pharmaceutical Sciences.* Utkal University, Bhubaneswar. Orissa. 751 004.

Shailendra Gurav., Vijay Gulkari., Nandkishore Duragkar and Arun Patil,2007. Pharmacognosy, phytochemistry, pharmacology and clinical applications of Gymnema sylvestre R Br. *Department of Pharmaceutical Sciences, Nagpur university*, Amaravati road, Campus, Nagpur 1(2):414-433.

Trease, G.E. and Evans, W.C.1996. Pharmacognosy. *Macmillan Publishers Ltd*. 213-832.

Zobel M.I., Glowniak K., Lynch J.E., Dudka S. and Alliota A.1999. *Chemistry, Trent University, Peterborough*, ON, Canada. K9J-7B8.

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Original Article

Effect of Plant Growth Regulator on *In vitro* Multiplication of Turmeric (*Curcuma longa* L. cv.Ranga)

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Abstract

High frequency *in vitro* plantlet regeneration method was developed for *Curcuma longa* L. (cv.Ranga) using fresh sprouting rhizome bud on semisolid culture media. The explants were cultured on Murashige and Skoog's (MS) medium supplemented with different concentration and combinations of BAP (6-Benzyl-amino-purine) and NAA(α- Naphthalene acetic acid) for shoot and root induction. Explants cultured on MS basal medium supplemented with 2.0mg/l BAP+0.5gm/l NAA showed highest rate of shoot multiplication. *In vitro* shoots were rooted on to the half-strength MS basal media supplemented with 2.0 mg/l NAA and rooting was better. Rooted shoots were transplanted in the green house for hardening and their survival rate was 95% in the field condition.
Keywords: *Curcuma longa* L. (cv.Ranga), Micropropagation, Plant growth regulator, Sprouting rhizome bud, Tissue culture

Introduction

Turmeric (*Curcuma longa* L. cv.Ranga) a perennial herb and an important spice, belong to family *Zingiberaceae*. The plant grown to a height of three to five feet, and is cultivated extensively in Asia, India, China, and other countries with a tropical climate. It has oblong, pointed leaves and bears funnel-shaped yellow flowers (Hand book of Horticulture, 2003). The rhizome is the portion of the plant used medicinally; it is usually boiled, cleaned, and dried, yielding a yellow powder. Dried rhizome of *Curcuma longa* is the source of the spice turmeric, the ingredient that gives curry powder its characteristic yellow color. Turmeric is used extensively in foods for both its flavor and color. Turmeric has a long tradition of use in the Chinese and Ayurvedic systems of medicine, particularly as an anti-inflammatory agent, and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic. Turmeric can also be applied topically in poultices to relieve pain and inflammation. Current research has focused on turmeric's antioxidant, hepato-protective, anti-inflammatory, anticarcinogenic, and antimicrobial properties, in addition to its use in cardiovascular disease and gastrointestinal disorders (Ammon and Wahl, 1991, Kiso *et al*.,1983).

The active constituents of turmeric are the flavonoid, curcumin and volatile oils including tumerone, atlantone, and zingiberone. Other constituents include sugars, proteins, and resins (Roses,1999). The best-researched active constituent is curcumin, which comprises 0.3 to 5.4 percent of raw turmeric. Pharmacokinetic studies in animals demonstrate that 40-85 percent of an oral dose of curcumin passes through the gastrointestinal tract unchanged, with most of the absorbed flavonoid being metabolized in the intestinal mucosa and liver. Due to its low rate of absorption, curcumin is often formulated with bromelain for increased absorption and enhanced anti-inflammatory effect. Curcumin, a major bio-reactive secondary metabolite obtained from the rhizome of turmeric, which is anticarcinogenic, is now being used in anticancer drug development programmes (Sakamura *et al*., 1986) Leaves and stems of turmeric are also used as biofertilizer. In India, ethnologically, turmeric occupies an important position in our life, forming an integral part of rituals, ceremonies and cuisine (Holttum,1950). In Orissa the turmeric cultivated variety Ranga of Kandhamala enjoys a monopoly in its production and export to different parts of the country. In conventional method turmeric is propagated vegetatively by perennial rhizomes. Low productivity, disease susceptibility and higher cost of seed rhizomes production are major constraints faced by the farmers. Turmeric rhizomes have a dormancy period and only sprout during monsoon. A large amount of the edible part (rhizome) is stored for stock purpose for the next season. Maintenance of germplasm by annual planting is expensive and labor intensive for the marginal farmers.

Moreover, diseases such as rhizome rot, caused by *Pythium* species and leaf spot, caused by species of *Taphrina* and *Collectrichum,* take a heavy toll during storage and in the field, there by causing a severe shortage of healthy planting materials.

In the recent decade's micro-propagation techniques are being profitably used to over come such constraints in various vegetatively propagated crop as well as ornamental and horticultural plants. Considering high demand and greater economic and medicinal value of turmeric, it is necessary to develop a suitable farmer friendly protocol for mass production of disease free stocks through tissue culture technique. There are many reports on *invitro* propagation of some rhizomatous plants like Ginger, Cardamom and *Alpania calcarata* (Yasuda et al.1987, 1988). The tissue culture protocols for turmeric have also been reported by few workers . How ever in Orissa no previous work was done on *invitro* propagation of Ranga cultivated variety of Turmeric. In the present study effort was made to establish a low cost *invitro* protocol for the Ranga cultivated variety of turmeric from sprouting rhizome bud. This cultivated variety of turmeric in Orissa has a special reputation both national and international market as a best condiment. This work is obviously a first step in the advancement of turmeric tissue culture in Orissa. It is therefore highly desirable to standardize a methodology for efficient *invitro* culture to provide a year round supply of disease-free quality planting materials for large scale commercial cultivation of the crop species to meet the market demand and socioeconomic development of the marginal farmers of the state.

Materials and Methods

Explant source

Healthy rhizome sprouts with active buds were collected from the rhizome of *Curcuma longa* (cv-Ranga) maintained in the nursery bed of experimental garden of P.G. department of Botany Utkal University. They were cut in to 1.5 to 2 cm length with active buds intact. These rhizome sprouting with active buds were washed with 5% (v/v) detergent solution Teepol (Qualigen, Mumbai, India) for 10 minute and rinsed several times with running tap water. These rhizome sprouting bud cuttings were surface sterilized with bavistin 0.3% followed by streptomycin 0.2% for 10 minute and then washed with sterile distilled water and transferred to laminar air flow cabinet .In the laminar chamber the sprouting bud cuttings were again dip with 70% alcohol for 30 second to one minutes followed by another treatment in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. Finally, the sprouting bud cuttings were washed thoroughly 3 to 4 times with sterile distilled water and soaked with sterile blotting paper and used as explants for *In vitro* cultures before the inoculation in to sterilized nutrient agar media pre-packed in culture tubes (Smith and Hamill,1996).

Culture medium and condition: The sterilized blotted explants were implanted on to the Murashige and Skoog's (1962) agar-gelled medium fortified with various concentrations/combinations of growth hormones. For shoot induction, the medium was supplemented with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l BAP and 0.25 to 0.5 mg/l α-Naphthalene acetic acid (NAA) either individually or in combination. For root induction *invitro* raised shoots measuring about 4-5cm grown in multiplication medium were cultured on half-strength MS medium supplemented with either NAA (α-Naphthalene acetic acid) or IBA (Indole3-butyricacid) in concentration of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l. The pH of the medium was adjusted to 5.8 before autoclaving at 1.04 kg/cm^{-2} pressure and 121°C temperature for 15 - 20 minute. Molten medium of 20 ml was dispensed into the culture tube and plugged with nonabsorbent cotton wrapped in one layer of cheesecloth. All cultures were incubated in 16 h light/8 h dark photoperiod (cool, white fluorescent light - 30µmol $m^{-2}S^{-1}$) . The cultures were incubated at 25 ± 3°C in diffused light under 60 - 70% relative humidity in the culture room. Each treatment had 20 culture tubes and the experiment was repeated thrice. The cultures were maintained by regular subcultures at 2 weeks intervals on fresh medium with the same compositions.

Acclimatization

Rooted micro-propagules were removed from the culture tube and the roots were washed under running tape water to remove agar .Then the plantlets were transferred to sterile poly pots (small plastic cups) containing pre-soaked vermiculite (TAMIN ,India) and maintained inside growth chamber

set at temperature 28^0C and 70-80% relative humidity. After three weeks they were transplanted to earthen pots containing mixture of soil + sand + manure (FYM) in 1:1:1 ratio and kept under shade house for a period of three weeks for acclimatization.

Observation of cultures and presentation of results: Twenty cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analyzed by the Post- Hoc Multiple Comparison test at the $P < 0.05$ level of significance (Marascuilo and McSweency, 1977).

Results

The response of *Curcuma longa* (cv.Ranga) rhizome sprouting bud explants cultured on different shoot proliferation media over a period of six weeks is presented in Table.1, culture medium devoid of growth regulators (Control, Table.1) failed to stimulate the bud break response in the explants even when the cultures were maintained beyond the normal observation period of four weeks. MS medium with growth regulator (BAP and NAA) supplements produced better results in terms of percentage explants response (Fig.2. A), shoots /explant, average shoot length and average number of roots and length of roots. In such media combinations bud break was noticed within 8-10 days of culture (Table.1, Fig.1. A&B). Of the combination tested MS + BAP (2.0 mg/l) + NAA (0.5mg/l) elicited optimal response in which an average of 7.0 ± 0.18, shootlets (Fig.1.C&D,Table-1) with a mean shoot length of 5.4 ± 0.09 cm per explant was recoded followed by the second best shoot multiplication 4.5 ± 0.12 in the medium MS + BAP (1.5 mg/l) + NAA (0.5mg/l) with a mean shoot length of 4.6 ± 0.16 cm . Higher concentration of BAP (3.0mg/l) with NAA (0.5 mg/l) showed callusing of explants with fewer number of shoots .In such cultures shoots were stunted with a mean shoot length of 2.4 ± 0.24 cm. The well developed elongated shoots measuring about 4-5cm in length were excised from shoot clump and transferred to half strength MS medium containing NAA or IBA. The rooting responses of shoots on different media, which included rooting percentage, days required for root initiation mean number of roots/shoot and mean root growth over a period of three weeks were different (Table-2.Fig.2.B). There was very stunted rooting in case of shoot planted on auxin free basal medium (control). Similarly, at lower level of NAA (0.25 mg/l) treatments there was hardly any rooting in the cultured shoots during the 4 weeks of observation period. However higher concentration of NAA (1.5&2.0 mg/l) and IBA at all concentration (i.e. from 0.25.0.05, 1.0, 1.5,2.0, 2.5 and 3.0 mg/l) tested responds well. Rooting was better in the culture which had combination of $^1/_2$ MS+2.0 mg/l NAA where about 95 % cultures responded with an average number of 7.3 ± 0.32 roots per plantlet and an average root length 4.5 ± 0.12 cm was recorded (Fig.1.C&D, Table-2) .The second highest response (80%) was recorded at 1.5 mg/l of NAA (Pillai and Kumar, 1982, Sugaya 1991, Zakaria and Ibrahim, 1986). It was observed that root primordial emerged from the shoot base starting from day 8 to 10 days after shoot inoculation and soon after that the root growth was rapid. NAA has more effective than IBA on induction of rooting as days required to rooting was only 8 to10 days as against the 10 to15 days required for similar response in case of IBA (Shirgurkar *et al*., 2001).

Discussion

The dependence of cultured explants on bud break response and shoot multiplication has already been established and extensively discussed (Babu *et al.*,1992). This has also been recently reported in the case of micro propagation of other Zingiberaceae like *curcuma longa* (Balachandran *et al*.,1990), *Zingiber officinale* (Hashim et al. 1998 Sharma and Singh 1994;1995, Bhat *et al*.,1994). In the present study, sprouting rhizome bud of *Curcuma longa* (cv. Ranga) showed significantly higher response in the medium with the combination of BAP (2.0 mg/l) + NAA (0.5mg/l). The quality of shoots and the over all growth response in terms of average shoot length was better in this growth regulator combination. A comparatively lower response was recoded when BAP was added alone in the medium. Review of literature indicates that the addition of either NAA or IBA or IAA in the culture medium improved the response in a number of species in terms of shoot growth. It has been reported that *Spathiphyllum floribundam* when cultured on media with BA supplement alone, a limited proliferation of explants with a maximum of average of 1.8 shoots per cultured explants was

observed ,while addition of IAA produced an average number of 11.6 shoots per explant (Ramierez-Magon et al. 2001). Similar observations were reported in *Hovenia dulcis* nodal culture (Echeverrigaray et al. 2001). In our study it was observed that addition of NAA 0.5 mg/l with BAP (2.0mg/l) showed improved response over BAP alone. Some authors also suggested that the combination of BAP and NAA were needed for producing more number of multiple shoots on *Curcuma longa* (Hoque *et al*.,1999, Hosoki *et al.*,1977; Noguchi *et al.*,1998).

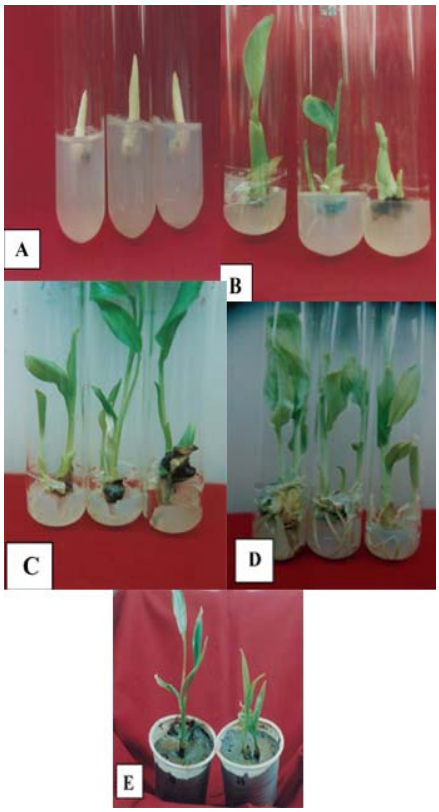

Fig.1:A-E,.Invitropropagation of *Curcuma longa* L. (cv-Ranga)
Fig.A. Rhizome sprouting buds are inoculated in shooting media; Fig. B. Shoot initiation in culture tube;Fig.C. Shoot lets in rooting media
Fig.D. In vitrogenerated shoot lets ready for hardening; g. E. Complete propagule in plastic pot after hardening

Rooting and establishment of plants in soil: Production of plantlets with profuse rooting in *invitro* is important for successful establishment of regenerated plants in soil (Sharma and Singh ,1994 ;1995; Sunitibala et al. 2001).The auxins NAA and IBA were used singly to induce rooting from *invitro* raised shoot lets. A range of concentration was tested (0.25,0.05,1.0,1.5,2.0, 2.5 and 3.0 mg/l) for rooting. In the present study $^{1}/_{2}$ strength MS basal medium and the two different auxins (NAA and IBA) were tried, the maximum results on rooting were obtained on half MS medium with supplementation of NAA (1.5&2.0 mg/l) than IBA (1.5&2.0 mg/l). Our observation are in accordance with the result of Inden et al. (2003) in *Z. officinale* and Salvi et al. (2000, 2001) in turmeric. The well rooted plants were transferred to plastic cups containing vermiculite for hardening and kept under controlled condition (Fig-E). Upon transferred to vermiculite medium plants started producing fresh shoots and roots after one week of transplantation. Later they were transferred to the field and the survival rate was 95% .The efficient micro-propagation technique described here may be highly use full for raising disease free quality planting material of *Curcuma longa* (cv. Ranga) for commercial and off season cultivation which not only help the socioeconomic development of the farmers but also fulfill the spice value and market demand including the conservation of genetic stock of the native species of Orissa for need of the hour.

Conclusion

In the present investigation the *in vitro* micro-plantlet multiplication system of cultivated variety Ranga of *Curcuma longa* has been optimized through rhizome sprouting bud as source of explant. However, our developed protocol can be used to produce a higher amount of large scale propagules as compared to previously reported protocols. Production of *in vitro* plantlets would be a suitable methodology for direct regeneration of shoot lets as a source of disease free quality planting material that could be stored and transported easily and a step forward towards commercial scale of propagule production in turmeric.

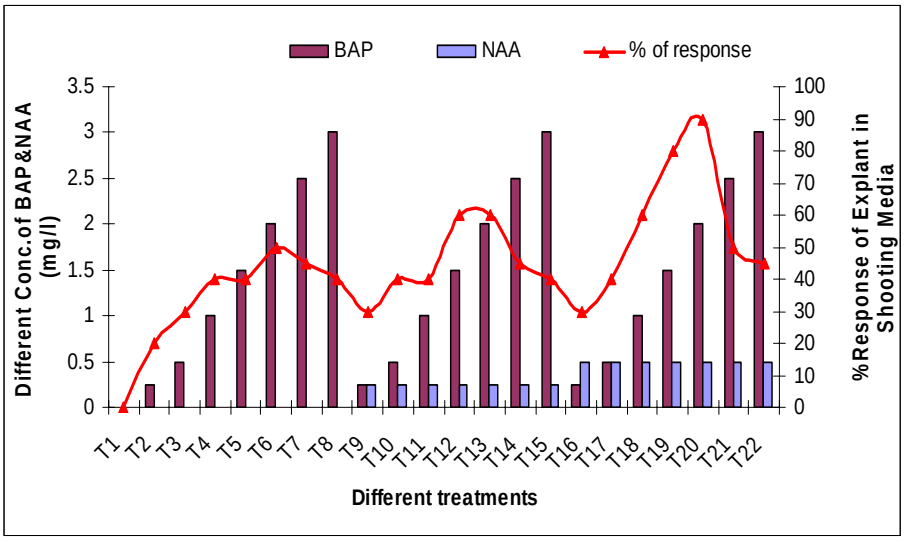

Figure-2A: Shooting Medium: Showing % of Explant Response in Different Treatments of BAP and NAA in MS medium.

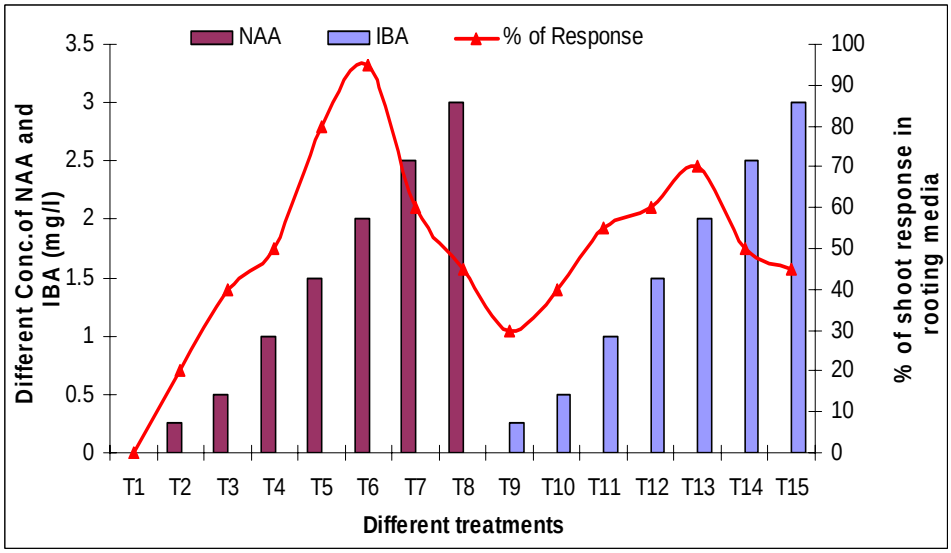

Figure-2B: Rooting Medium: Showing % of Micro shoots Response in Different Treatments of NAA and IBA in ½ MS medium

Table-1: Shoot formation in rhizome sprouting of *Curcuma longa* L. (cv.Ranga) cultured on semisolid MS medium supplemented with various concentration of BAP and NAA [20 culture tube per treatment, data scored after 6 weeks]

Different treatments	Growth Regulators(mg/l)		% of Explant response	Days to bud break	Mean No of shoot/explant ±S.E.	Mean shoot length(cm) ±S.E.	Mean No of nodes/shoot ±S.E.
	BAP	NAA					
T1	0	0	-	-	-	-	-
T2	0.25	0	20	12-15	2.0 ± 0.17+	1.1 ± 0.14+	1.0 ± 0.09+
T3	0.5	0	30	12-15	2.5 ± 0.04+	1.6 ± 0.08+	1.6 ± 0.20+
T4	1.0	0	40	12-15	2.5 ± 0.23+	2.0 ± 0.14+	2.1 ± 0.14+
T5	1.5	0	40	12-15	2.6 ± 0.29	2.6 ± 0.09	2.0 ± 0.20
T6	2.0	0	50	12-15	2.8 ± 0.14	2.6 ± 0.04	2.3 ± 0.22
T7	2.5	0	45	14-16	2.2 ± 0.26+	2.8 ± 0.08+	2.2 ± 0.16+
T8	3.0	0	40	14-16	2.0 ± 0.18+	2.5 ± 0.33+	2.0 ± 0.24+
T9	0.25	0.25	30	12-15	2.2 ± 0.09+	2.5 ± 0.12+	1.5 ± 0.09+
T10	0.5	0.25	40	12-15	2.7 ± 0.04+	2.8 ± 0.24+	2.3 ± 0.17+
T11	1.0	0.25	40	10-12	3.1 ± 0.16+	3.1 ± 0.32+	2.0 ± 0.14+
T12	1.5	0.25	60	10-12	3.3 ± 0.14	3.0 ± 0.36	2.5 ± 0.12
T13	2.0	0.25	60	12-15	3.2 ± 0.32	3.2 ± 0.16	2.8 ± 0.12
T14	2.5	0.25	45	12-15	2 .4 ± 0.30+	2.6 ± 0.24+	2.8 ± 0.21+
T15	3.0	0.25	40	14-16	2.0 ± 0.21+	2.4± 0.34+	2.6 ± 0.32+
T16	0.25	0.5	30	12-15	2.2 ± 0.28+	2.1 ± 0.20+	2.4 ± 0.12+
T17	0.5	0.5	40	12-15	2.3 ± 0.14+	2.2± 0.12+	2.6 ± 0.08+
T18	1.0	0.5	60	10-12	3.5 ± 0.41+	3.1 ± 0.33+	3.1 ± 0.24+
T19	1.5	0.5	80	8-10	4.5 ± 0.12	4.6 ± 0.16	3.5 ± 0.08
T20	2.0	0.5	90	8-10	7.0 ± 0.18	5.4 ± 0.09	4.0 ± 0.28+
T21	2.5	0.5	50	10-12	3.5 ± 0.08+	3.2 ± 0.18+	3.0 ± 0.09+
T22	3.0	0.5	45	10-12	3.2 ± 0.16+	2.4 ± 0.24+	2.6 ± 0.20+

[20 culture tube per treatment; repeated thrice. Means are calculated by Post-Hoc Multiple Comparisons tests at $P < 0.05$ level of significance ,+ callusing at the basal end , S.E.:-Standard error of mean]

Table-2:Influence of different levels of NAA and IBA on rooting response of *in vitro* generated shoot lets of *Curcuma longa* L.(cv.Ranga) [20culture tube /treatment ,data scored after 4 weeks]

Different treatments	Growth regulators augmented with1/2 strength MS basal medium(mg/l)		% of Explant Response	Days to root initiation	Mean root numbers ±S.E.	Mean root length (cm) ±S.E.
	NAA	IBA				
T1	0	0	-	-	-	-
T2	0.25	0	20	12-15	1.1 ± 0.12+	1.0 ± 0.09+
T3	0.5	0	40	10-12	2.1 ± 0.12+	2.0 ± 0.09+
T4	1.0	0	50	10-12	2.6 ± 0.09+	2.2 ± 0.20+
T5	1.5	0	80	8-10	5.0 ± 0.20	3.5 ± 0.16
T6	2.0	0	95	8-10	7.3 ± 0.32	4.5 ± 0.12
T7	2.5	0	60	10-12	4.2 ± 0.34+	3.2 ± 0.24+
T8	3.0	0	45	10-12	3.2 ± 0.18+	2.6 ± 0.28+
T9	0	0.25	30	12-15	1.0 ±0.04+	1.5 ± 0.04+
T10	0	0.50	40	12-15	2.3 ± 0.12+	2.3 ± 0.12+
T11	0	1.0	55	10-12	2.4 ± 0.04+	2.3 ± 0.14+
T12	0	1.5	60	10-12	2.5 ± 0.28	2.4 ± 0.18
T13	0	2.0	70	10-12	2.8 ± 0.12	2.6 ± 0.12
T14	0	2.5	50	12-15	2.6 ± 0.16+	2.3 ± 0.20+
T15	0	3.0	45	12-15	2.5 ± 0.32+	2.0 ± 0.09+

[20 culture tube per treatment; repeated thrice. Means are calculated by Post-Hoc Multiple Comparisons tests at $P < 0.05$ level of significance ,+ callusing at the basal end , S.E.:-Standard error of mean]

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References

Ammon, H. P. T. and Wahl, M.A. 1991. Pharmacology of *Curcuma longa*. *Planta Med*., 57: 1–7.

Balachandran, S.M., Baht, S.R. and Chandel, K.P.S.1990. *In vitro* clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc.). *Plant Cell Reports*, 8: 521-524.

Babu K.N., Samsudeen, K. and Ratnambal, M.J. (1992). *In vitro*plantregenaration from leaf-derived callus in ginger. *Plant Cell Tiss. Org.Cult*. 29: 71-74.

Bhagyalakshmi, A. and Singh, N.S.1998. Meristem culture and micropropagation of a variety of ginger (*Zingiber officinales* Rosc.) with a high yield of oleoresin. .*J.Hort.Sci*. 321-327.

Bhat, S.R., Chandel, K.P.S. and Kacka, A.1994. *In vitro* induction of rhizomes in ginger (*Zingiber officinale)* Rosc. Indian J Exp. Biol. 32, 340-4.

Echeverrigaray, S., Mossi, A.Z. and Munari, F.1998. Micropropagation of raisin tree (*Hovenia dulcis* Thunb.) through axillary bud culture. *J. Plant Biochem.Biotechnol*.7: 99-102.

Hashim, H.M., Ibrahim, H. and Rahim, Z.1998. Preliminary studies on some nutritional composition of the edible gingers. In Advance in Biochemistry and Biotechnology in Asia and Oceania. *Procedings of the 7th Federation of Asia and Oceania Biochemists Symposium* (FAOB). El-2.

Holttum, R.E. 1950. The Zingibeareacea of the Malay Peninsula. *Gard. Bull.Sing*. 13: 1-250.

Hoque, M.I., Perveen, S. and Sarker, R.H.1999. *In vitro* propagation of Ginger.(*Zingiber officinales* Rocs.) *Plant tissue cult*. 9: 45-51.

Hosoki, T. and Sagawa, Y.1977. Clonal propagation of ginger (*Zingiber officinale* Rocs.) through tissue culture. *Hort.Sci*.,12: 451-452.

Inden, H., Hirano, A. and Asahira, T. 1998. Micropropagation of ginger. *Acta. Hortic*, 230: 177-184.

Kiso, Y., Suzuki, Y., Watanabe, N., Oshima, Y. and Hikino H.1983. Antihepatotoxic principles of *Curcuma longa* rhizomes. *Planta Med*., 49: 185–187.

Marascuilo, L.A. and Mcsweeney, M.1977. Post-Hoc Multiple Comparisons in sample preparations for test of homogenesity, p. 141-147. *In* M. McSweeney & L.A. Marascuilo (eds.). Non-parametric and distribution free methods the social sciences. Books/Cole, U.S.A.

Murashige, T. and Skoog, F.1962. A revised medium for rapid growthand bioassays with tobacco tissue cultures. *Physiol Plant*, 15: 473-497.

Noguchi, Y. and Yamakawa, O. 1998. Rapid clonal propagation of ginger(*Zingiber officinales* Rosc.). *Japan J.Breed*.,38: 437-442.

Pillai, S.K. and Kumar, K.B. 1982. Note on the clonal multiplication of ginger *in vitro*. *Indian J. Agric. Sci.* 52: 397-399.

Ramirez-Magon, R., Borodanenko, A., Barrera-Guerra, J.L. and Ochoaalejo, N. 2001. Shoot number and shoot size as affected by growth regulators in in vitrocultures of *Spathyphyllum floribundum* L. *Scin. Horti.* 89: 227-236.

Roses, I.A.1999. Medicinal Plants of the World. Chemical Constituents, Traditional and Modern Medicinal Uses. Humana Press, Totowa, New Jersey.

Sakamura, F., Ogihara, K., Suga, T., Taniguchi, K. and Tanaka, R. 1986. Volatile constituents of *Zingiber officinale* rhizomes produced by *in vitro* shoot tip culture. *Phytochemistry*,25: 1333-1335.

Sharma, T.R. and Singh, B.M .1994. Production of disease-free encapsulated buds of *Zingiber officinales* Rosc. *Plant Cell Rep*. 13: 300-307.

SHARMA, T.R. and SINGH, B.M.(1995). *In vitro* microrhizome production in *Zingiber officinales* Rosc.Plant cell Rep.15, 274-277.

Shirgurkar, M.V., John, C.K. and Nadgauda, R.S. 2001. Factors affecting *in vitro* micro-rhizome production in turmeric. *Plant Cell, Tissue and Organ Cult*. 64(1): 5-11.

Smith, M.K. and Hamill, S.D.1996. Field evaluation of micropropagated and conventionally propagated ginger in subtropical Queensland. *Australian J Exp Agricul*, 36: 347-54.

Sugaya, A. 1991. Micropropagation of turmeric (*Curcuma domestica Valet*) and other *Curcuma* species. *Biotechnology in Agriculture and Forestry*. 19, 277-94.

Sunitibala, H., Damayanti, M. and Sharma, G.J. 2001. *In vitro* propagation and rhizome formation in *Curcuma longa Linn. Cytobios,* 105 (409): 71-82.

Yasuda, K.T. Tsuda and Sugaya, A. 1987. Multiplication of *Curcuma* species by tissue culture. *Planta Medica*. 54: 75-9.

Zakaria, M., and Ibrahim, H.1986. Phytochemical screening of some Malaysian

species of Zingiberaceae. *Malaysian Journ. of Sci.* 8: 125-128.
Salvi, N.D.,George, L. and Eapen, S. 2000. Direct regeneration of shoots from immature inflorescence cultures of turmeric. *Plant Cell Tiss. Org. Cult*. 62: 235-238.
Salvi, N.D., George, L. and Eapen, S. 2002. Micropropagation and field evaluation of micropropagated plants of turmeric. *Plant Cell Tiss. Org. Cult*. 68: 113-119.
Yasuda, K.T. Tsuda. and Sugaya, A. 1988. Multiplication of *Curcuma* species by tissue culture. *Planta Medica*, 54: 75-79.

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Original Article

In vitro multiple shoot induction through axillary bud of *Ocimum basilicum* L. an important medicinal plant

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Abstract

Micropropogation of *Ocimum basilicum* L. a medicinal plant known for its traditional and folk remedy to treat various ailments, has been carried out by using axillary explants on Murashige & Skoog's medium. Nodal explants produced proliferation of multiple shoots on the medium containing 0.5 mgl^{-1} BAP with 0.5 mgl^{-1} IAA. The elongated shoots were separated and cultured for root induction. Rooting of *in vitro* raised shoots were best induced on ½ strength MS medium supplemented with 1.5 mgl^{-1} IBA with highest percentage of shoot regenerating roots (89 %). The well rooted plantlets were acclimatized and successfully established in the natural condition with 90% survival.

Keywords: *Ocimum basilicum,* Medicinal plants, Regeneration, Nodal segments, Shoot tip

Introduction

Ocimum basilicum L. (Sweet basil) is a small perennial, culinary herb tropically growing shrub of Asian origin (Dhar, 2002). It is widely cultivated for the production of essential oils, and also marketed as an herb, either fresh, dried, or frozen (Putievsky *et al*., 1999). The essential oil of sweet basil possesses antifungal, insect-repelling and toxic activities (Reuveni *et al*. 1984; Dube *et al*. 1989; Werner *et al*., 1995). Chiang *et al.,* 2005 has also reported antiviral activities. The leaves and flowers of sweet basil are traditionally used as antispasmodic, aromatic, carminative, digestive, galactogogue, stomachic, and tonic agents (Lust, 1983; Chiej, 1984; Duke *et al*., 1985; Sahoo *et al.*, 1997; Phippen and Simon, 1998). *Ocimum basilicum* is also a globally important economic crop producing annually ca. 100 tonnes of essential oil worldwide and with a trade value as a pot herb of around US $ 15 million per year. It is also widely used in systems of indigenous medicine (Paton 1996). Usually the plant is regenerated through seeds and creeping stem nodes. But due to the indiscriminate collection of huge amount of this plant by local herbalists and Ayurvedic and Unany companies, this plant species is on the verge of extinction. Under such a situation it is important to develop techniques for rapid mass propagation of this species to meet up the commercial need and also for protecting the genetic erosion. *In vitro* micropropagation is an effective mean for rapid multiplication of species in which it is necessary to obtain a high progeny uniformity. Therefore, the interest in using these techniques for rapid and large-scale propagation of medicinal and aromatic plants has been significantly increased (Sahoo *et al.*, 1997).

In vitro micropropagation technique has been proved to be employed in propagation of many of medicinal plant species (Sivakumar and Krishnamurthy 2000, Selvakumar *et al*., 2001, Wawrosch *et al*., 2001, Das and Handique 2002, Kalidass *et al.,* 2008, Kalidass and Mohan 2009). Many *in vitro* studies have been conducted on Lamiaceae species, including the *Ocimun* genus, using different explants, like nodal segments (Shahzad and Siddiqui, 2000; Begun *et al.*, 2002), leaf explants (Phippen and Simon, 2000), young inflorescence (Singh and Sehgal, 1999) and axillary buds (Begum *et al.*, 2002).

Materials and Methods

Nodal segments from natural population of *Ocimum basilicum* were collected and used in the present study (Fig 1a). Nodal segments with a single axillary bud were used as explants. The explants were washed under running tap water, pre-soaked in liquid detergent for about 20 – 30 min, and surface sterilized using 70% (v/v) ethanol for 1 min and 0.1% (w/v) mercuric chloride for 2-5 min. It is then

washed with sterile double distilled water. The surface sterilized explants were sized to 1cm length contain a single node with an axillary bud. The explants were inoculated vertically on the culture medium. Shoot proliferation and adventitious shoot regeneration were achieved on Murashige & Skoog's (1962) basal medium supplemented with different concentration and combination of BAP, IAA & NAA (Table 2). The regenerated shoots were excised aseptically with the help of sterile scalpel under laminar air flow cabinet. The shoots were then inoculated on half strength of MS medium with different concentrations and combinations of IBA (indole 3-butyric acid) and NAA (napththaleneacetic acid) for root induction. Both proliferation and rooting media contained 3% sucrose and gelled with 6 % agar (Hi-Media, India). The pH was adjusted to 5.7±0.1. All media were steam sterilized under 1.1 kg/cm^2 pressure at 121°C. Cultures were grown at 25±1 °C under 16 h photoperiod with a light intensity of 2000 – 3000 lux. The well rooted plantlets were gently removed from the culture tubes without any damage, washed to remove agar and media adhered to the roots and transplanted to plastic pots filled with soil and compost (1:1) for hardening. The plantlets were kept in a polychamber at 90% relative humidity, 30±1°C under a 24h photoperiod for acclimation. Established plants were transplanted in earthen pots under natural conditions and the survival rate was recorded.

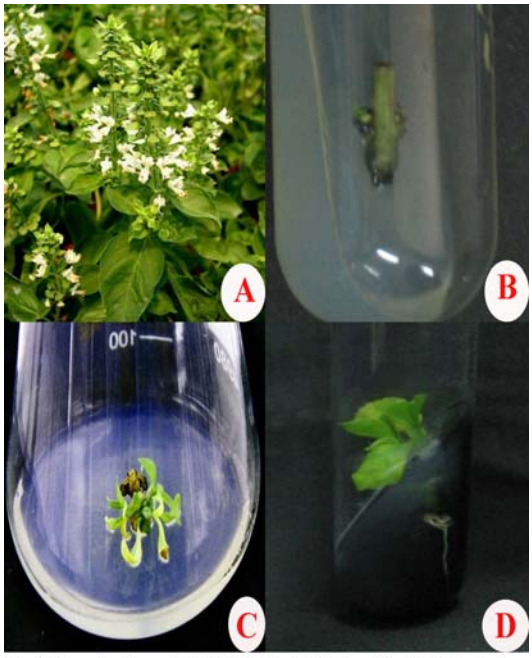
Results and Discussion

In multiple shoot proliferation nodular explants response better than other explants viz, leaves and inter nodes for this reason all experiments were carried out using nodal segments. Similar finding of auxiliary buds proliferation have also been reported in many medicinal plants (Anand and Jeyachandran 2004; Hassan and Ray 2004; Kalidass and Mohan 2009). The nodal explants under direct organogenesis on MS supplemented with various concentration and combinations of BAP, NAA and IAA were studied (Table 1). The best response with maximum shoot elongation was obtained using 1.0 mgl^{-1} BAP in combination with 0.5 mgl^{-1} IAA after four weeks of culture. Our results also show that 1.0 mgl^{-1} BAP with 0.5 mgl^{-1} IAA promotes formation of high multiple shoots (Fig 1c). *O. basilicum* shows good response towards plant regeneration in MS medium in the presence of BAP combined with auxins as reported by various authors (Sahoo et al., 1997; Begum et al., 2002; Phippen and Simon 2000 & Dode et al., 2003). The role of BAP and IAA in shoot formation has also been recorded in other medicinal plants (Arockiasamy et al., 2002; Marta *et al*., 2009 and Taware *et al*., 2010).

Table 1. Effect of different concentrations and combinations of growth regulators on MS medium for the adventitious shoot regeneration from the nodal explants of *Ocimum basilicum.*

S. No	Growth regulators (mg/l)			Shooting response (%)	Mean No. of shoots (Mean ± SE)	Mean length of shoots (cm) (Mean ± SE)
	BAP	IAA	NAA			
1	0.5	0.0	0.0	30	8.6 ±0.11	1.8± 0.21
2	1.0	0.0	0.0	42	7.9± 0.07	1.5± 0.14
3	1.5	0.0	0.0	46	9.8± 0.12	2.3± 0.17
4	2.0	0.0	0.0	28	6.2±0.08	6.6± 0.22
5	2.5	0.0	0.0	31	6.4±0.16	2.5± 0.16
6	3.0	0.0	0.0	24	7.3± 0.24	5.9± 0.26
7	0.5	0.5	0.0	71	12.5± 0.11	5.3± 0.10
8	1.0	0.5	0.0	82	23.8± 0.23	6.8± 0.14
9	1.5	0.5	0.0	64	13.8± 0.07	3.5± 0.13
10	2.0	0.5	0.0	48	12.9± 0.09	2.4± 0.17
11	2.5	0.5	0.0	56	12.8± 0.06	1.9± 0.09
12	3.0	0.5	0.0	62	14.6± 0.16	1.6± 0.11
13	0.5	0.0	0.5	52	21.6± 0.24	4.9± 0.21
14	1.0	0.0	0.5	46	12.3± 0.09	3.2± 0.14
15	1.5	0.0	0.5	49	13.5± 0.06	2.6± 0.18
16	2.0	0.0	0.5	40	10.7± 0.12	2.5± 0.29
17	2.5	0.0	0.5	44	11.6± 0.23	2.2± 0.26
18	3.0	0.0	0.5	36	7.3± 0.16	1.9± 0.18

After four weeks, the well developed shoots were transferred to half strength MS medium supplemented with IBA singly and in combination with NAA (Table 2). In different concentration of IBA tested, 1.5 mgl^{-1} IBA in half strength MS was found to be most suitable for root induction (Fig 1d). The supplementation of auxin either singly or in combination was also reported in many plant species (Gopi et al. 2006; Baksha *et al*., 2007; Kalidass *et al*., 2008; Kalidass and Mohan, 2009). However, the addition of IBA also favored rooting in other medicinal plants like *P. kurrooa* (Chandra *et al*., 2006), *S. cordifolia* (Sivanesan and Jeong, 2007a), *P. indicum* (Sivanesan and Jeong, 2007b), and *W. somnifera* (Sivanesan, 2007).



A - *Ocimum basilicum* L. habit, B - Shoot initiation, C - Multiple shoots, D - Rooting of *Ocimum basilicum* L.

For acclimatization, plantlets were removed from rooting medium after three weeks of incubation and transferred to plastic pots containing autoclaved soil rite covered with perforated polythene bags to maintain humidity and were kept under culture room conditions for one week. Then they were planted under normal garden conditions. After hardening the growth rate of the plantlets was slow initially and increased gradually. New leaves emerged from the hardened plantlets after three weeks. Most of the plantlets (90 %) survived after hardening.

Table 2: Effect of different concentration of half strength of MS medium on root induction of *in vitro* shoots.

S. No	Growth regulators (mg/l)		Rooting response (%)
	IBA	NAA	
1	0.5	0.0	56.25±1.41
2	0.0	0.5	45.71±1.72
3	0.1	0.0	47.14±2.56
4	0.5	0.0	62.50±3.10
5	1.0	0.0	81.25±0.83
6	**1.5**	**0.0**	**89.00±0.73**
7	2.0	0.0	60.00±1.22
8	1.0	0.5	51.25±2.03
9	1.5	0.5	38.88±2.02
10	2.0	0.5	00.00±0.00

About 90% of the regenerated plantlets could tolerate and survive under *ex vitro* environment or field conditions. A number of plantlets were lost due to damping off and necrosis during acclimatization in *ex vitro* condition. Loss of regenerants due to such symptoms was also observed in *Eucalyptus tereticornis* (Gill et al. 1993), *Solanum nigrum* (Ara et al. 1993), *Rauvolfia serpentine* (Ilahi 1993) and *Rosa damascena* (Kumar et al. 1995). This study supports the rapid multiplication of this useful medicinal plant by *in vitro* conditions. This report provides a simple protocol for the micropropagation of *O. basilicum*. The shoots can be easily derived from node cultures on BAP and IAA containing medium and subsequently rooted on IBA containing medium. This protocol could be utilized for conservation and clonal propagation of this economically important medicinal plant.

References

Anand, S.P and Jeyachandran, R. 2004. *In vitro* multiple shoot regeneration from nodal explants of *Zehneria scabra* (L.f.) Sonder - An important medicinal climber. *Plant Tissue Cult.* 14: 101-106.

Ara, M., Jahan, A and Hadiuzzaman, S. 1993. *In vitro* plant regeneration from leaf explant of *Solanum sisymbrifolium* Lamk in Bangladesh. Intl. Plant Tissue Cult Conf., Dhaka, 19 - 21 Dec. pp. 47.

Arockiasamy, DI., Muthukumar, B., Natarajan, E and John Britto, S. 2002. Plant Regeneration from Node and Internode Explants of *Solanum trilobatum* L. *Plant Tissue Cult.* 12(2): 93-97.

Baksha, R., Miskat Ara Akhter Jahan., Rahima Khatun and John Liton Munshi. 2007. *In vitro* Rapid Clonal Propagation of *Rauvolfia serpentina* (Linn.) Benth *Bangladesh J. Sci. Ind. Res.* 42(1): 37-44.

Begum, F., Amin, N., Azad M.A.K. 2002. *In vitro* rapid clonal propagation of *Ocimum basilicum* L. *Plant Tissue Culture*, 12:27-35.

Chandra, B., Palni, L.M.S, Nandi, S.K. (2006). Propagation and conservation of *Picrorhia kurrooa* Royle ex Benth.: an endangered Himalayan medicinal herb of high commercial value. Biodivers. Conserv. 15: 2325-2338.

Chiang, L.C., Cheng, P.W., Chiang, W., Lin, C.C.2005. Antiviral activity of extracts and selected pure constituents of *Ocimum basilicum. Cli Exp Pharmacol Physiol.* 32: 811-816.

Chiej, R. 1984. Encyclopedia of medicinal plants. MacDonald : ISBN 0-356-10541-5.

Das, J and Handique, P.J. 2002. Micropropagation of a rare medicinal plant species *Polygonum microcephallum* D.Don., through high frequency shoot multiplication. *J. Phytol. Res*. 15(2): 197-200.

Dhar, A.K.2002. Sweet Basil: *Ocimum basilicum*- a review. Journal of Medicinal and Aromatic Plant Sciences. 24:738-55.

Dode, L.C., Bobrowski, V.L., Braga, E.J.B., Seixas, F.K., Schunch, W. 2003. *In vitro* propagation of *Ocimum basilicum* L. *Maringa*. 25: 435-437.

Dube, S., Upadhyay, P.D., Tripathi, S.C. 1989. Antifungal, physicochemical, and insect repelling activity of the essential oil of *Ocimum basilicum, Can. J. Bot.* 67: 2085–2087.

Duke, J.A and Ayensu, E.S. 1985. Medicinal Plants of China. Reference Publications. Inc. : ISBN 0-917256-20-4. Reference Publications, Algonac, MI (USA).

Gill, R.I.S., Gill, S.S and Gosal, S.S.1993. Vegetative propagation of *Eucalyptus tereticornis* Sm. through tissue culture. Intl. Plant Tissue Cult. Conf., Dhaka 19 - 21 Dec., pp. 44.

Gopi, C., Nataraja Sekhar, Y and Ponmurugan, P. 2006. *In vitro* multiplication of *Ocimum gratissimum* L. through direct regeneration. *African Journal of Biotechnology Vol.* 5 (9), pp. 723726

Hassan, A.K.M.S and Roy, S.K. 2004. Micropropagation of *Smilex zeylanica* L., a perennial climbing medicinal shrub, through axillary shoot proliferation. *Bangladesh J. Life Sci*. 16(1): 33-39.

Ilahi, I.1993. Micropropagation and biosynthesis of alkaloid by *Rauvolfia* cell culture. Intl. Plant Tissue Cult. Conf., Dhaka 19 - 21 Dec. pp. 21.

Kalidass, C and Mohan, V.R. 2009. *In vitro* rapid clonal propagation of *Phyllanthus urinaria* Linn. (Euphorbiaceae)- A medicinal plant. *Researcher 1*(*4*): 56-61.

Kalidass, C. Manickam, V.S and Glory, M. 2008. In vitro studies on Leptadenia reticulata (Retz.) Wight & Arn. (Asclepiadaceae). *Indian Journal of Multidisciplinary Research*, 4(2): 221 – 225.

Kumar, S., Choudhary, M.L and Raghava, S.P.S. 1995. *In vitro* manipulation of *Rosa damascena* Mill for oil contents. 2nd Intl. Plant Tissue Cult. Conf., Dhaka, 10 – 12 Dec., pp. 47.

Lust, J. 1983. The Herbs book. Bantan Books: ISBN 0-553-23827-2.

Marta Valdez Melara, Andrés M. Gatica Arias. 2009. Effect of BAP and IAA on shoot regeneration in cotyledonary explants of *Costa rican* melon genotypes, *Agronomía Costarricense* 33(1): 125-131.

Murashige, T and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 443-477.

Paton, A.1996. Basil Taxonomy, Kew Scientist 9 : 7

Phippen, W.B and Simon J.E. 2000. Shoot regeneration of young leaf explants from basil (*Ocimum basilicum* L.), In Vitro Cell. Dev. Biol. Plant, Largo, 36, 250-254.

Phippen, W.B., Simon, J.E. 1998. Anthocyanins in basil. *J. Agric. Food Chem.,* Washington, DC., v. 46, p. 1734-1738.

Putievsky, E and Galambosi, B. 1999. Production systems of sweet basil, in: R. Hiltunen, Y. Holm (Eds.), Basil. The Genus *Ocimum*, Harwood Academic Publishers, pp. 39–65.

Reuveni, R., Fleischer, A and Putievsky, E. 1984. Fungistatic activity of essential oils from *Ocimum basilicum* chemotypes, Phytopath. Z. 110: 20–22.

Sahoo, Y., Remien, Y.N., Yao, R.S.1997. *In vitro* clonal propagation of an aromatic medicinal herb *Ocimum basilicum* L. (sweet basil) by axillary shoot proliferation. *In vitro Cell. Devel. Biol. Plant, Largo*, 33, 293- 296.

Selvakumar, V., Anbudurai, P.R and Balakumar, T. 2001. *In vitro* propagation of the medicinal plant *Plumbago zeylanica* L. through nodal explants. *In Vitro Cell Dev Biol. Pl. 37*: 280-284.

Sharzad, A., Siddiqui, S.A.2000. *In vitro* organogenesis in *Ocimum sanctum* L. – A

multipurpose heb. *Phytomorphology*, Delhi, v. 50, n. 1, p. 27-35.

Singh, N.K., Sehgal, C.B.1999. Micropropagation of "Holy basil" (*Ocimum sanctum* L.) from young inflorescens of mature plants. Plant Growth Regul., Dordrecht, v. 29, p. 161-166.

Sivakumar, G and Krishnamurty, K.V. 2000. Micropropagation of *Gloriosa superba* L. an endangered species of Asia and Africa. *J. Curr. Sci*. 78(1): 30-32.

Sivanesan I, Jeong BR (2007b). Micropropagation and *in vitro* flowering in *Pentanema indicum* Ling. *Plant Biotechnol*. 24: 527-532.

Sivanesan, I and Jeong, B.R. 2007a. Direct shoot regeneration from nodal explants of *Sida cordifolia* Linn. *In Vitro Cell. Dev. Biol. - Plant* 43: 436-441.

Sivanesan, I. 2007. Direct regeneration from apical bud explants of *Withania somnifera* Dunal. *Indian J. Biotechnol*. 16: 125-127.

Sultana, S and Handique, P.J. 2004. Micropropagation of *Wedelia chinensis* through high frequency shoot multiplication using nodal explants. *J. Curr. Sci.* 5: 447-452.

Taware, A.S., Mukadam, D.S., Chavan, A.M and Tware, S.D. 2010. Comparative studies of in vitro and in vivo grown plants and callus of Stevia rebaudiana (Bertoni). *International Journal of Integative Biology*. 9(1): 10 – 15.

Wawrosch, C., Malia, R.R and Kopp, B. 2001. Clonal propagation of *Lilium nepalense* D. Don, a threatened medicinal plants of Nepal. *Plant Cell Rep*. 10: 457-460.

Werner, R.A. 1995. Toxicity and repellency of 4-allylanisole and monoterpenes from white spruce and tamarack to the spruce beetle and *Eastern larch* beetle (Coleoptera: Scolytidae), *Environ. Entomol.* 24: 372–379.

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Original Article

Micropropagation and *in vitro* flowering in *Solanum nigrum* linn. A medicinal plant

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Abstract

A protocol was developed for rapid multiplication of *Solanum nigrum* a medicinal plant, through *in vitro* culture of mature nodal explants. Multiple shoots were induced on both Murashige & Skoog's medium supplemented with varying concentrations and combinations of auxins and cytokinins (α-naphthaleneacetic acid, indolyl-3 acetic acid, 6-benzyladenine and kinetin). Maximum number of shoots was developed with medium fortified with 13.5 μM BAP. For rooting of the excised shoots were rooted on half strength woody plant medium supplemented with IAA 5.58 μM and IBA 4.92 μM were used. Thus, a reproducible protocol has been established for micropropagation of this species.

Keywords: *Solanum nigrum*; Multiple shoots; BAP; IAA; Murashige & Skoog's medium; axillary shoot

Introduction

Solanum nigrum is an erect annual herb. The juice of the plant is diuretic and used to cure chronic enlargement of liver, piles, dysentery and fever (Kumar *et al*., 1997). The drug made from this plant acts as laxative, improve appetite and this is administered against asthma, leprosy skin diseases (Bhattacharjee 2001). Due to large scale and unprohibited exploitation of the natural resource by the pharmaceutical industry, the wild stock of this medicinally important plant has been markedly depleted. *S. nigrum* can be propagated by seeds and vegetative cuttings. Root behaviour of stem cutting and non availability of seeds due to over exploitation are major get back for plant propagation processes are season dependent and can be achieved only during monsoon period. The application of *in vitro* techniques might be of great value as an alternative method to achieve rapid multiplication independent of Season (Fay,1994).

In the present study, a reliable protocol has been developed for large scale propagation of this important medicinal plant using multiple axillary shoot proliferation from single node cultures is described. There is no earlier report on *in-vitro* micropropagation of this useful plant.

Materials and Methods

Plants of *S. nigrum* were collected from Tirunelveli hills of Southern Western Ghats and established in the herbal garden and green house attached to the Centre for Biodiversity and Biotechnology, St. Xavier's College, Palayamkottai. Young shoots (5cm length) were collected from the green house. Plants were defoliated and washed in running tap water for 10 min. Surface decontamination of the shoots consisted of passage through 0.1% $HgCl_2$ (w/v) for 1½ min and three washes in sterilized distilled water. Then single nodes of 1-1.5 length were dissected and inoculated aseptically on to Murashige & Skoog's (1962)solidified with agar 0.5%,Himedia,Mumbai) and supplemented with 3% of sucrose and various concentration and combinations of plant growth regulators, viz 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA).

The pH of the medium was adjusted to 5.8 before adding 0.5% W/A agar and autoclaved at 121°C for 15 minutes in Astell (Scientific - U.K.) Autoclave. All the cultures were incubated at 25° ± 2°C under cool white fluorescent tubes (1500 - 2000 lux) for 16 hours/days. After few weeks , the multiple shoots formed, were cultured onto root inducing medium ½ M.S supplemented with 2% of Sucrose and different concentration of Auxin Indole-3 Butric Acid (IBA), Indole – 3 Acetic Acid (IAA) and NAA. After the shoot formation the *in vitro* raised plantlets were removed from culture tube, washed thoroughly in running tap water before transplanting into small polycups containing mixture of sterilized sand and garden soil and irrigated with 1/10 diluted liquid MS medium and covered with poly bags for *in vitro*

hardening. After hardening in polycups they were subsequently transferred to 15 cm diameter pots containing, sand and compost (2:1:1) and maintained under mist irrigation. Then they were shifted for field planting. Experiments were performed with a minimum of 15 replicates for initiation and were repeated at least twice.

Results and Discussion

When MS medium supplemented with different concentration of BAP was used, multiple shoots emerged from the nodal explants after 12 days of inoculation. The effect of BAP on shoot multiplication from nodal explant is shown in Table 1. The medium containing 13.5 μM of BAP induced multiple shoots (3-5) with maximum percentage of responding cultures (75%). The concentration of BA at 0.88 μM was too low to induce only 1 shoot / node.

Shoot elongation did not correlate well with shoot production with short length and number of nodes / shoot got consistently declined with increasing concentration (22.2 μM) of BAP. Perusal of literature suggests that BAP as a cytokinin is most active at concentration in many plant systems (Scott *et al*., 1995, Kathiravan & Ignacimuthu 1999). Therefore the maximum caulogenic response observed in this study at low concentration (0.44 μM) together with a decreasing response at concentration exceeding 8.88 μM is somewhat different. Similarly though the negative influence of BA at higher concentration on shoot and inter nodal length is known (Emmanuel *et al*. 2000). Hence the optimal shoot production at 0.44 μM BAP was achieved with less than satisfactory shoot elongation and number of node formation. Maximum number of flowers was also obtained on the MS medium supplemented with 2.22 μM of BAP. Shoot elongation can be achieved by transferring shoot clusters to a fresh medium with decreased concentration of cytokinin or by substituting less active cytokinin at dark conditions, the one which was employed for shoot induction (George,1996).

Flowering was considered to be a complex processes regulated by both internal and external factors and its induction under *in vitro* culture is extensively rare (Stephan and Jayabalan, 1998).

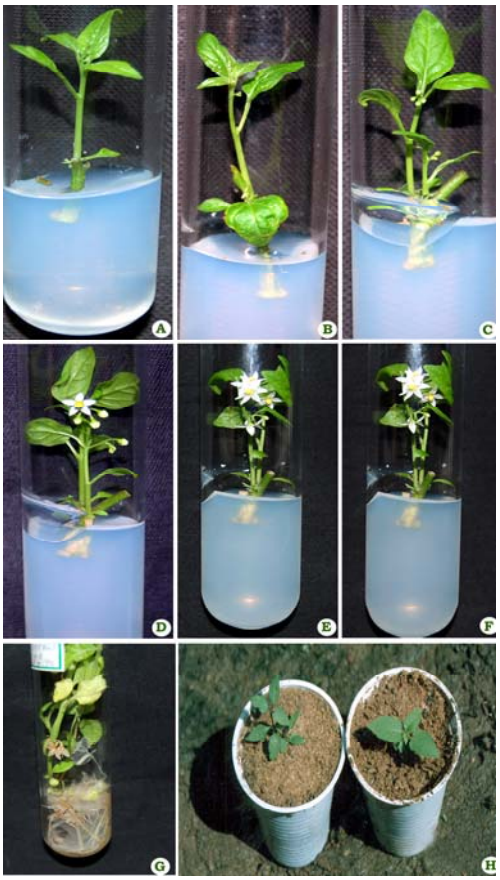

Fig.1: Micropropagation stages of *Solanum nigrum* Linn.
A – C: Shoot formation from nodal segment of *Solanum nigrum* on M.S Medium containing BAP & NAA; D - F. Flower bud initiation; G. Rooting of shoots on MS Medium Containing IBA;Transplanted plant in pot of *Solanum nigrum;* Hardened plantlets of *Solanum nigrum*

In vitro flowering was observed within 15 days of Culture (Fig. 1 D –F). When in vitro shoots, derived from nodal explants were transferred to rooting medium. For in vitro flowering, the response the response of IBA and NAA is better than IAA. High frequency and maximum number of flowers per explants was more on MS-medium supplemented with 0.8mg/lof IBA and 0.3mg/l of NAA compare to other concentration. Auxin support in vitro flowering, similar results were observed by patil *et al*., (1993) in Sun Flower, Vandana *et al*.,(1995)in Cauli flower, Naik and Lata (1998) in Coriander . Cytokinin did not support in vitro flowering either singly or in combination with auxins.

Table- 1: Effect of BAP on shoot production from the nodal segments of S*olanum nigrum* L.on ms medium

Sl. No.	BAP concentration (μM/*l*)	Percentage of shooting	No. of shootlets/ node ± SD (after 5 weeks of culture)	Mean length of shootlets (cm)
1	0.44	60	1.58 ± 0.06	2.36±0.185
2	0.88	65	1.46± 0.13	1.62±0.189
3	2.22	72	1.67 ± 0.32	1.95±0.404
4	4.44	75	1.56 ± 0.33	2.20±0.496
5	8.88	74	1.70 ± 0.41	2.03±0.536
6	13.50	75	1.57 ± 0.21	2.07 ±0.500
7	17.75	74	1.59 ±0.42	2.00±0.489
8	22.20	72	1.57 ± 0.48	2.02±0.459

Each experiment was performed with 10 replicates and was repeated thrice.

Table- 2: Effects of auxins on rooting of *in vitro* shoots of S*olanum nigrum* L..in half strength ms medium

Concentration μM/*l*			% of rooting response	Mean no. of roots / shootlets ± S.D.	Mean length of rootlets (cm)
IAA	IBA	NAA			
5.58	4.92	-	79	3.35 ± 0.30	1.42± 0.19
1.12	9.84	-	57	1.35 ± 0.22	1.39± 0.20
-	19.68	-	60	2.02 ± 0.51	2.03± 0.17
-	0.98	21.48	62	2.46 ± 0.21	1.78± 0.93
1.12	4.92	-	61	1.37 ± 0.23	1.51± 0.03
-	9.84	11.16	61	1.70 ± 0.20	1.44± 0.20
-	14.76	1.12	62	2.19 ± 0.24	1.97± 0.57
-	19.68	1.12	62	1.45 ± 0.28	1.14± 0.17

Each experiment was performed with 10 replicates and was repeated thrice.

The *in vitro* raised multiple shoots were excised and transferred individually to half strength MS medium supplemented with varied concentration of the root inducing auxins IBA, NAA and IAA. IBA, IAA was more effective than NAA in inducing robust roots in shoot cultures. Of the concentration tested, IAA (5.58μM) IBA (4.92 μM) gives maximum number of rootlets and root length were also observed in the same concentration of IAA, IBA. The percentage of rooting response was also the highest (79%). These results are in accordance with those of Mustafa Anand *et al*. (1997) on *Kaempteria rotunda*, Manickam *et al*. (2000) on *Withania somnifera* (Indian ginsens) and Segio *et al*. (2000) on *Anthemis robilis*. When IAA was tested for rooting, there was not only a decrease in the rooting response but also enhance the callus formation. Plantlets(30 day old) were transferred to polycups contain the mixture of soil and vermiculite(1:1) for hardening. After 4 weeks 25 *in vitro* rooted plantlets were transferred to pots and to the field after few weeks. The survival percentage was 65%. The present investigation has resulted in a protocol which could be used for mass propagation of *Solanum nigrum* to meet the increasing demand of the pharmaceutical industry as well as for conservation of this important medicinal plant.

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References

Anwar Shahzad Hashma Hasan and Saeed, A Siddiqui, 1999. Callus induction Regeneration in *Solanum nigrum* L. *In vitro*. *Phytomorphology*. 49 (2) : 215 -220.

Bhattacharjee, S.K.2001. Hand book of medicinal plants. P.326.

Emmanuel, S. Ignacimuthu, S. and Kathiravan, K 2000. Micropropagation of *Wedelia calendulaceae* Less., A medicinal plant; *Phytomorphology*, 50: 195-201.

Fay, M. F.1994. In what situations is *in vitro* culture appropriate to plant conservation Biodiversity and Conservation 3: 176-183.

Jabeen, F.T.Z Venugopal,R.B.G.,Kiran Kavinraj and Srinath rao,2005.Plant rengeneration and *In vitro* Flowering from leaf and nodal explants of *Solanum nigrum* (L.) – An important Medicinal Plant, *Plant Cell and Molecular Biology*, 6 (1&2) : 17-22.

George E F Plant propagation by Tissue Culture part 2, 2nd edn. (Etegetics Ltd. Edington, U.K.) 1996.

Katheravan,K and Ignacimuthu, S,1999. Micropropagation of *Canavalia virosa* (Roxb.) Wight & Arn. A medicinal plant, *Phytomorphology*, 49: 61-66,

Kumar, A. S. and Kumar, A. 1995. Plant regeneration from cultured embryonic axis in *Thevetia peruviana* L. *Indian Expl. Biol*. 33: 190-193.

Manickam,V.S. Elangomathavan, K. and Antonysamy, R.2000. Regeneration of Indian ginseng plantlets from stem callus, *Plant cell Tissue Org. Cult*. 62: 181-185

Murashige, T. and Skoog, F.1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures, physiologia Pl. 15: 473-479.

Mustafa, A. and Molly, H.1997. *In vitro* multiplication of greater Galangal (*Alpinia galanga* Linn. Wild) - a medicinal plant, *Phytomorphology*, 47: 45-50.

Nair, G .R. and Lata, K.1996. *In vitro* flowering in *Morus alba* L. (Mulberry) Bull. Of purr.Sure and applied Sci, 15 : 129-132.

Patil, M. S. Ramaswamy, N. M. and Sree Rangasamy, S. R.*1993. In vitro* flowering in sunflower (*H. annuus* L.) *Curr.Sci*. 65:565-566.

Scott, E. S. Rao, A. N. and Loh, C.S.1995. Preliminary studies on Micropropagation of *Hopea odorata* a depterocarp tree, *Plant Cell Tiss. Org. Cult*. 41: 193-196.

Segio, E. and Simone, B. 2000. Clonal propagation of Roman chanamile 1998) (*Anthemis nobilis* L.). *J. Herbs. Spices and medicinal plants* 7: 35-41.2000.

Stehen R and Jayabalan, N,1998. *In vitro* flowering and seed setting formation of Coriander (*Coriander sativum* L.) *Current Sci*. 74 (3) : 195-197.

Vandana, A. Kumar, Atul Kumar, and Jitendra Kumar,1995. *In vitro* flowering and pod formation in cauliflower (*Brassia oleracea* var. *Botrytis*). *Curr.Sci*. 543 -545.

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Original Article

Clonal propagation of *Adiantum capillus* – veneris

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Abstract

Adiantum capillus - veneris is an important medicinal fern in South India. In the present study, establishment of protocol for its mass propagation of *A. capillus* - veneris was initiated using spores as an explant. Explants were cultured on MS basal medium and were incubated in the dark at 22±2°C. After 12 weeks germination of spores, calli and prothalli were obtained. After germination, cultures were transferred on to fresh medium every 4-week. Prothalli were sub-cultured on MS medium supplemented with BAP (0.01-2 mg^{-1}/L), 2,4-D (0.1-1.5 mg^{-1}/L) and combination with BAP and 2,4-D where they multiplied successfully. Callus induction and protonema formation was achieved in higher percentage in case of MS medium supplemented with 1.5+2.0 μM^{-1}/L 2,4-D+BAP and 0.75+1.50 μM^{-1}/L 2,4-D+IBA and 4% glucose. They were sub-cultured into phytohormone-free MS medium with 4% glucose, finely cultured ferns were transferred to poly cups and maintained for 15 days in green house condition.

Key words: Maiden hair fern, *Adiantum capillus* - veneris, spores, calli

Abbreviations: MS- Murashige and Skoog Medium; BAP – Benzylaminopurine; 2,4-D; 2,4-Dichlorophenoxy acetic acid, PGRs – Plant Growth Regulators

Introduction

Mass propagation of plants through *in vitro* culture is one of the best and most successful option of commercial application of plant tissue culture technology. The first successes in the field of the intensive multiplication of plants through *in vitro* techniques are cited around 1970, the fern *Nephrolepis exaltata bostoniensis* being the first plant micropropagated *in vitro* with a commercial purpose (Cachita-Cosma and Dorina,1987). Recently, there has been much progress in this technology for some medicinal plants (Bertrand *et al*., 1999, Fernandez *et al*.,1999). Tissue culture propagation and its importance in conservation of genetic resources and clonal improvement have been described by many workers (Barz *et al*., 1977; Datta and Datta,1985;Kukreja *et al*., 1989; Jusekutty *et al*., 1993).

Ferns, a lower group of plants having a rich source of medicines which are used as decoction or infusion to ease labor pains. These plant extracts used as depurative, to reduce fever and as a hair wash (Burkhill,1935).

The genus *Adiantum* (L.) belongs to the family Adiantaceae, which consists of 150 to 200 species worldwide distributed in North America, United States, South Dakota, British Columbia, Canada and India (Fernald, and Lyndon,1950; Hickman,1993; Paris and Cathy,1993; Lellinger and David,1985; Gleason and Cronquist,1991;Victor *et al*.,2003). Whole plants of *A. capillus*-veneris are used as tonic for cough, throat infection, visual tumours and menstrual problems (Guhabaskhi *et al*.,1999). Active constituents include 21-OH-adiantone, isoquercitrin, kaemferol, letuol, terpenoids, 3α-4α-poxyfilicane, flavones tannic acid, gallic acid and essential oils were reported to be responsible for the potent medicinal values of this fern (Irudayaraj and Patric Raja,1998; May,1978). Victor *et al*., (2003) reported the antimicrobial activity of leaves and pinnae oils. In the present study a rapid protocol for *in vitro* mass production of *A. capillus* - veneris through high-frequency calli from spore explants followed by successful establishment of regenerated fern was achieved.

Materials and Methods

Fern material

Fresh plant (fern) material was collected from Aluthakanni River, Tenkasi, Tirunelveli District, Tamil Nadu, South India.

Spore collection

Spores were collected from *A. capillus*- veneris fronds and dried on filter paper in an oven at 30ºC for three days. The spores were separated from sporangia by filtering through tissue paper, and were stored in glass jars under refrigeration at 7 ± 1 ºC.

Spores sterilization

Spores of *A. capillus*- veneris were sterilized in 2% sodium hypochlorite for 10 minutes, filtered through sterile filter paper by vacuum, washed several times with sterile distilled water and dried in a laminar flow hood for 30 min (De Brum and Randi, 2002).

In-vitro culture medium

The culture medium used in the present study was Murashige and Skoog (1962) basal medium with 3% (w/v) sucrose and 0.8% (w/v) agar. The medium was further augmented with different concentrations of BAP (0.01–2.0µM), 2, 4-D (0.01 – 2.0µM) separately and in combination in the concentration range of 0.01–2.0µM (Table 1). Spores of *A. capillus* - veneris were aseptically inoculated in MS basal medium on 100ml sterile bottle. Spore germination in different stages like protonema stage, gametophyte, young sporophytes formation was observed at a temperature of 20±5^{o}C and at a light intensity between 3-15 µMolm^{-2}s^{-1}. Morphogenetic response was examined at the given cultured condition. Germinated aseptic cultures were transferred into fresh media in six weeks interval. Further, the *in vitro* developed shoots were transferred into rooting medium supplemented with 2,4-D 0.01-1.0µM/L in combination with 0.01-2.0µM/L IBA. After root development, regenerated plantlets were transferred to pots for hardening.

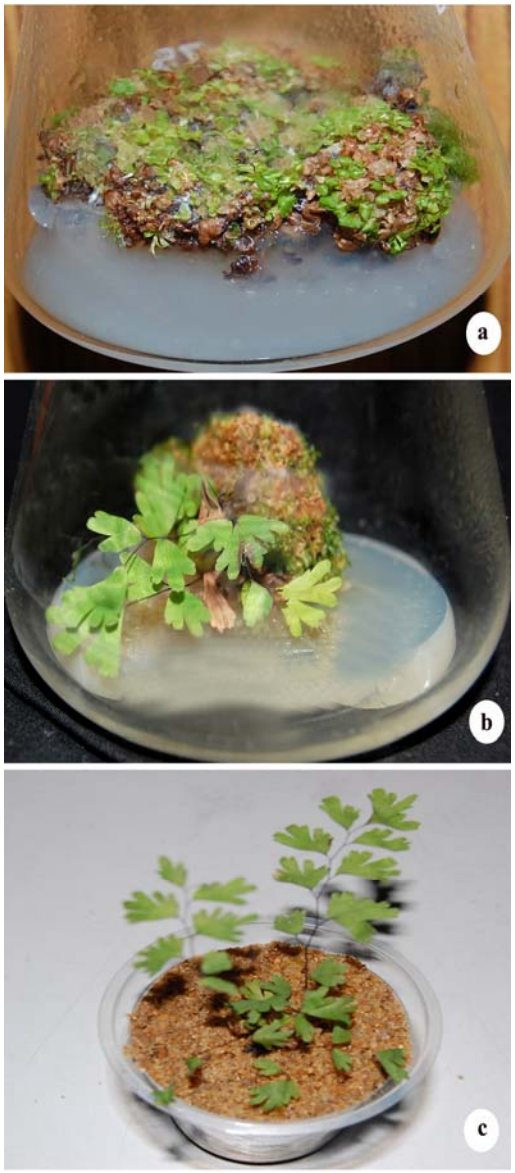
Results and Discussion

The protocol for *in vitro* culture of *A. capillus* - veneris, is summarized in Fig.1. The germination of spore started within 3-5 days. Spores have been used as the explant source for successful high frequency regeneration of plants.

Although the regeneration of plants from spores is quite difficult *in vitro*, optimization of every step from initiation to acclimatization makes it more feasible to produce plants from spores (Banks,1999). Due to the phenomenon of vegetative reproduction of the gametophyte, frequently noticed *in vitro*, a high frequency of regeneration has been obtained, a fact that is also reported by Fernandez *et al*.,(1999). The excellent capacity of *in vitro* multiplication of the gametophyte can however influence the number of the sporophytes formed, as was noticed in *Asplenium*, *Dryopteris*, *Osmunda*, etc., as the absence of the sporophyte presupposes a nutritional competition between the two generations. This presupposition is supported by the fact that, when the multiplication of the gametophyte is blocked in *Osmunda*, the production of sporophytes increases (Fernandez *et al*., 1999).

Successful spore culture establishment in *Asplenium nidus* was reported recently (Khan *et al*.,2008). The callus and dermal hair were developed within 60-70 days. After 70-100 days, the callus were elongated and bulged. In 100-120 days of culture maintenance, embryo development started, which resulted in the formation of sporophytic leaf (Fig.1). Table 1 shows the responses obtained when the spores of *A. capillus* - veneris were cultured on MS medium supplemented with different concentrations of 2,4-D/ BAP in combination. Medium containing 1.5 and 2.0 µM/L BAP showed the best frequency of calli production rates and also BAP (2.0µM/L) in combination with 2,4-D (1.5µM/L) and 0.75+1.50µM/L 2,4-D+IBA. This observation authenticate that plants need both auxins and cytokinins which should be supplied *in vitro* and the ratio between auxin and cytokinin seems to be very important for multiplication (Bertrand *et al*., 1999, Fernandez *et al*., 1999).

There are many reports in which application of growth regulators were highlighted towards either enhanced or suppressed plant growth which directly influence the rate of multiplication (Fernandez and Revilla, 2003). The *in vitro* regeneration of Pteridophyta is currently used for the mass multiplication of the ornamental and medicinal species, and also for the endangered ones, with a view to preserving them *ex situ*.



While, in the spore culture, only one prothallus is, as a rule, obtained from a single spore, in the case of the green sporangia culture, from a single explant a colony is obtained, formed numerous prothalli, on account of the fact that, in various manners, the secondary gametophytes are formed (Soare,2008). The experiments of growth with isolated prothalli and pairs of prothalli have shown that, on average, 56% of the isolated prothalli are successful in forming the sporophyte and the percentage is much larger in the case of the pairs of prothalli, which indicates the fact that a crossed fecundation, between the gametes on different prothalli, is produced in *Asplenium trichomanes* (Suter *et al*., 2000). In the present study it was possible to obtain a higher multiplication of prothalli and further propagation, hardening, maintenance, hardening and field transfer is underway. This approach could be a vital one for the *ex situ* conservation of this medicinally important fern species and similar this may be extended for such similar species too.

Fig 1 (a). Prothalli growth on MS medium
(b). Subcultureed on calli forming prothallus with young sporophytes
(c). Hardening of *A. capillus* -veneris

Table -1. Frequency of callus induction, protonema regeneration and root induction of *A. capillus* -veneris in different concentrations of PGRs

PGRs	Concentration (µM)	No. of Explants	No. of calli (%)	No. of protonema	Frequency of callus induction
	0.01	93	0	93	0.0
	0.02	94	0	94	0.0
	0.05	91	0	91	0.0
	1.00	92	0	92	0.0
2,4-D	1.50	93	0	93	0.0
	2.00	91	0	91	0.0

PGRs	Concentration (µM)	No. of Explants	No. of calli (%)	No. of protonema	Frequency of shoot induction
BAP	0.01	92	75	21	81.52
	0.02	95	79	16	83.16
	0.05	94	76	15	80.85
	1.00	95	81	11	85.26
	1.50	91	84	8	92.31
	2.00	92	87	6	94.67
2,4-D+ BAP	0.01 + 0.01	91	79	16	86.81
	0.02 + 0.02	95	81	13	85.26
	0.05 + 0.05	93	82	9	88.17
	1.00 + 1.00	92	85	8	92.39
	1.50 + 1.50	94	89	5	94.68
	1.50 + 2.00	95	90	4	94.74

PGRs	Concentration (µM)	No. of Explants	No. of new Shoots	No. of Roots	Frequency of root induction %
2,4-D + IBA	0.01 + 0.01	91	10	3	30
	0.02 + 0.02	95	10	3	30
	0.03 + 0.05	93	10	5	50
	0.05 + 1.00	92	10	7	70
	0.75 + 1.50	94	10	8	80
	1.00 + 2.00	95	10	7	70

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References

Fernondez, H. and Revilla, M.A. 2003. *In vitro* culture of ornamental ferns. *Plant Cell. Tissue and Organ Culture*, 73:1-13.

Burkhill, I.H. 1935. *A Dictionary of the Economic Products of Malay Peninsular*. Ministry of Agriculture Malaysia, 1542.

Fernald and Lyndon M. 1950. *Adiantum capillus-veneris* in the United States. *Rhodora.* 52(620): 201-208.

Gleason, H.A. and Cronquist, A. 1991. Manual of the vascular plants of northeastern United States and adjacent Canada, Second Edition. Botanical Garden, New York, 16.

Hickman, and James, C. 1993. *The Jepson Manual*: Higher plants of California. Berkeley, California: University of California Press.1400.

Lellinger, and David, B.1985. A Field Manual of the Ferns and Fern Allies of the United States and Canada.: Smithsonian Institution Press, Washington, D.C. 389.

Paris and Cathy, A. 1993. *Adiantum* Linnaeus. *In* Flora of North America north of Mexico, Volume 2. Oxford University Press, New York, NY. 125-127.

De Brum, F.M.R. and Randi, A.M. 2002. High irradiance and temperature inhibit the germination of spores of the fern *Rumohra adiantiformis* (Forst.) Ching (Dryopteridaceae). *Revista Brasileira de Botanica*, 25:391-396.

Guhabaskhi, D. Sensarma, P. and Pal D.C.1999. A Lexicon of Medicinal Plants in india, Darbari off set, PVT, Ltd, Dixone Lane,Culcutta,1:54.

Irudayaraj, V. and Patric Raja, D.1998. Phytochemical studies on Indian Ferns. *Indian Fern.Journal*.15:149-168.

May, L.W.1978. The economic uses and associated folklore of ferns and fern allies. *Bot. Rev.*44:491-538.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.*15: 473-497.

Barz, W. Reinhard, E. and Zenk, M.H. 1977. Plant tissue culture and its Biotechnological Application. Springer-Verlag, Berlin, New York. pp. 27-43.

Datta, P.C. and Datta, S.C. 1985. Applied Biotechnology on Medicinal, Aromatic and

TimberPlants. Calcatta University, Culcatta, India.

Kukreja, A.K. Mathur, A.K. Ahuja, P.S. and Thakur, R.S. 1989. Tissue Culture and Biotechnology and Aromatic Plants. ICSIR, Lucknow, India.

Jusekutty, P.C. Swati, S. and Prathapasenan, G. 1993. Direct and indirect organogenesis in *Coccinia indica*. *J. Hort. Sci*. 68: 31-35.

Bertrand, A.M. Albuerne, M.A. Fernandez, H. Gonzalez, A. and Sanchez-Tames, R.1999. *In-vitro* organogenesis of *Polypodium cambricum. Plant Cell Tissue and Organ Culture*. 57: 65-69.

Fernandez, H. Bertrand, A.M. and Sanchez-Tames, R.1999. Biological and nutritional aspects involved in fern multiplication. *Plant Cell Tissue and Organ Culture*.56: 211-214.

Khan, S. Raziq, M. and Kayani, H A.2008. *In vitro* propagation of bird's nest fern (*Asplenium nidus)* from spores. *Pak. J. Bot*.40(1): 91-97.

Victor, B. Maridass, M. Ramesh, U. and Prabhu, J.M.A. 2003. Antibacterial activity of essential oils from the leaves of *Adiantum capillus* – veneris Linn. *Malaysian Journal of Sciences.* 22: 65-66.

Banks, J.A. 1999. Gametophyte development in Ferns. *Annual Rev. Plant Physiology*. 50:163-86.

Cachita Cosma and Dorina,1987. Metode *in vitro* la plantele de cultura - baze teoretice si practice, Edit. Ceres, Bucuresti, 274.

Soare,2008. *In- vitro* development of gametophyte and sporophyte in several fern species Liliana ristina. *Not. Bot. Hort. Agrobot. Cluj*. 36(1): 13-19.

Sutter, M. Schneller, J. and Vogel, J.J. 2000. Investigations into the genetic variation, population structure and breeding system of the fern *Asplenium trichomanes* subsp. *quadrivalens*. *Int. J. Plant. Sci*. 161 (2): 233-244.

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Original Article

Estimation of culturable microbes present in heavy metal contaminated and non contaminated Agricultural soil

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Abstract

Environmental contamination and exposure to heavy metals such as mercury, cadmium and lead is a serious growing problem throughout the world. Human exposure to heavy metals has risen dramatically in the last 50 years as a result of an exponential increase the agricultural land were affected seriously. Based on the analysis of AAS five samples were showed positive for the heavy metal contamination. Estimation of Culturable microbes present in the agricultural soil showed wild difference between the heavy metal contaminated and non contaminated rhizosphere soil. The maximum microbial load was observed in the non contaminated soil sample NCS1 ($26x10^8$ CFU ml^{-1}) and contaminated soil sample CS5 ($18x10^3$ CFU ml^{-1}). Thus heavy metals used in the industries like fireworks and match work will affect microbial load present in soil of nearby agricultural land.

Keywords: Heavy metals; pollution; Culturable microbes

Introduction

Due to urbanization and land degradation, the area of agricultural land is continuously decreasing. Proficient use of available agricultural land resources is important to overcome the deficiency. Soil is a non-renewable dynamic resource and acts as an interface between agriculture and the environment (Abou *et al*., 2008). Maintaining soil quality is the vital factor to improve crop yield and productivity. Among the soil quality maintance heavy metals plays an imperative role to sustain its eminence properties (McGrath *et al*. 1995; Cheng 2003). In India many industries are using heavy metals in their process and exiled out without proper treatment. Metals are released into the environment leads wide spectrum of anthropogenic activities such as smelting of metallic ores, industrial fabrication and commercial application of metals, which are polluting our aquatic bodies.

Though, several metals are essential for biological systems and must be present in a certain concentration range. Too low concentrations lead to a decrease in metabolic activity (Vajpayee *et al*.,1995). The fireworks that are displayed in the skies to celebrate events such as Independence Day and New Years Eve, etc.contain carcinogenic sulphur-coal compounds. Fireworks spread an odor of black gunpowder and spreads radioactive barium, which makes the green sparkling color and considerable amounts of strontium, along with arsenic, mercury, cadmium, lead, copper, zinc and chromium. (Patrick, 2003). Such a pollution of the environment by toxic metals and radionuclides arises as a result of many human activities, largely industrial, although sources such as agriculture and sewage disposal also contribute. These contaminations not only affect the human and also alter the microbial community structure. (Weis, 2004). Thus, finally this may also seriously affect the nearby agricultural land and food cultivate in this. In Sivakasi area, fireworks and match work industries are predominant one and the explosion of fireworks has been discovered to be a source of intense heavy metal release. Our aim is to compare and explore the microbial load in agriculture nearby industry.

Materials and Methods

Study Area

Sivakasi is well known for crackers, printing and Match factories. The area of investigation lies in and around Sivakasi agricultural area located near fire work factory. It is located approximately in 9.28' North latitude and 77.48' East longitude. The elevation of the area of investigation is 100.07 meter above sea level. Variation in the altitude and rain fall has a bearing on the vegetation in general.

Microbial analysis of heavy metal contaminated soil

Total Culturable bacteria in the normal agricultural and heavy metal contaminated soil samples were homogenized in phosphate buffered saline solutions and serially diluted and plated on Nutrient Agar medium. After 24 h incubation at 30 °C, plates were scored for their load in terms of Colony Forming Unit (CFU) and expressed it in lane values.

Screening of heavy metals in agriculture soil

Soils samples were taken from different site of agricultural area present in Sivakasi. Plant roots were taken by loosening the soil around the root and then gently removing the plant material from the ground. The root adhering soils were used to analyze for heavy metal presence. Heavy metals were analyzed by using 20 g of soil was mixed in 100 ml of distilled water and 0.5 ml was used to find in Atomic Absorption Spectroscopic (AAS). (Sinha *et al*., 1993).

Results

Soil characteristics of pH

Soil samples from various sites of agricultural lands present near the industry areas were taken for soil analysis. From the soil pH analysis 80% of the samples showed the pH ranged from 7.2 to 8.21 and remaining 20% of them in acid soils pH<6.7. This indicates that the pH for agricultural soils in Sivakasi has a tendency to be higher than natural and acidic soils pH. Organic matter and sand, silt, and clay contents vary significantly among soil samples (data not shown).

Soil organic contents

Organic content of the soil varies from soil to soil and it is highly influenced by the microbial community and cultivated plants. In this present study the level of organic soil ranges 0.9 to 12 %.

Soil conductivity (EC)

Electrical conductivity (EC) is a measurement of the dissolved material in an aqueous solution, which relates to the ability of the material to conduct electrical current through it. Actual conductivity of the soil is highly influenced by the nutritional measurements for fertilizers may vary due to the solubility and purity of the particular fertilizer source. Monitoring the changes in the nutrient solution over time will indicate what adjustments should be made to keep the solution in balance for the crop being grown. This principle can become more useful by determining for each crop the relationships among total dissolved solids, electrical conductivity, and concentration of each essential element and stage of plant growth under similar light conditions. In this study the soil have 15 to 39% soil conductivity (Table 1). Among them 10 samples taken from the agricultural soil having >30% conductivity and 6 of them were >20% and reaming were <20% conductivity.

Screening of Heavy metal presence in agricultural soils

Total concentration of heavy metals in the agricultural soils reflects a difference in the degree of soil contamination; the percentage of studied soils with concentrations of Cd, Cu, Ni, Pb and Zn. The concentrations of Cd, Ni, and Zn in the soils of the agricultural areas near to industries were significantly higher than the concentration of these same elements in the agricultural soils far to industries. The results (Table 2) also revealed a high variability of concentration of Cd, Ni, and Zn in different land uses. The concentration of the Nickel and cadmium was higher (3.9 and 4.1 ppm respectively) in sample 1 (S1) than remaining four samples (S2, S3, S4 and S5). Similarly the concentration of the zinc was higher in sample 3 (S3) than others.

Estimation of microbial biomass in Lead and Nickel contaminated soil

Microbial load present in and around the plant roots are varied based on environmental factor like heavy metals. In this study the microbial load of culturable bacteria present in this soil also varied from contaminated and non contaminated soils. (Mohammad Iqbal *et al*., 2008).The microbial load present in the non contaminated soil sample was high than the contaminated soils. The maximum load was observed in NCS1 sample ($26x10^8$ CFU ml^{-1}). Similarly the result of microbial load was coincided with the level of heavy metal concentration. (Phipps 1981).

Sample 3 (CS3) ($11X10^3$ CFU ml^{-1}) and Sample 5 (CS5) ($18X10^3$ CFU ml^{-1}) showed degreased level of bacteria compared to other three samples (CS1, CS2, and S4) (Table 3).

Table-1: Soil characteristic feature of agriculture soil samples taken from Sivakasi

Sample	Soil pH	Organic C content	Soil conductivity (% dw)
S1	8.5 ± 0.1	0.9 ± 0.25	35.8 ± 0.7
S2	8.4 ± 0.2	10.6 ± 0.18	36.8 ± 0.7
S3	7.9 ± 0.1	12.3 ± 0.34	29.0 ± 0.8
S4	6.3 ± 0.3	3.5 ± 0.68	15.8 ± 0.9
S5	7.8 ± 0.1	11.5 ± 0.45	37.1 ± 0.7
S6	6.3±0.2	0.9 ± 0.23	22 ± 0.30
S7	7.6±0.1	11.3 ± 0.45	34 ± 0.26
S8	7.2±0.2	11.9 ± 0.33	35 ± 0.88
S9	8.7±0.1	6.7 ± 0.69	19.5 ± 0.5
S10	7.5±0.2	1.7 ± 0.68	27.1 ± 0.6
S11	6.2 ± 0.1	10.8 ± 0.34	25.3 ± 0.76
S12	7.6 ± 0.2	1.3 ± 0.78	16.8 ± 0.55
S13	7.2 ± 0.1	12.2 ± 0.76	39.0 ± 0.9
S14	7.7 ± 0.3	5.5 ± 0.63	19.8 ± 0.5
S15	7.2 ± 0.1	11.4 ± 0.54	27.4 ± 0.4
S16	6.6±0.2	8.8 ± 0.23	32.6 ± 0.6
S17	7.6±0.3	11.1 ± 0.65	33.2 ± 0.7
S18	7.1±0.2	11.0 ± 0.32	24.7 ± 0.5
S19	7.4±0.2	4.6 ± 0.61	35.3 ± 0.7
S20	6.7±0.3	3.3 ± 0.50	32.7 ± 0.2

Values are represented as mean ±SD of triplicate

Discussion

Soil characteristics

Soil samples from various sites of agricultural lands present near the industry areas were readily contaminated with the materials used in that particular industries. In the fire industries most of the metals which were used to make colours are heavy metals. Thus in this studies attempts were taken to analysis. In the initial analysis of soil pH, organic matters and electric conductivity analysis revealed that most of the soils are coming under alkaline condition. Though it is alkaline, most of soils harbours good organic content and it is suitable for the cultivation. (Slobodkin, 2005).

The variation between the soils are highly influenced the microbial community and cultivated plants. In this present study the level of organic soil ranges 0.9 to 12 % is an average one. Electrical conductivity (EC) is a measurement of the dissolved material in an aqueous solution, which relates to the ability of the material to conduct electrical current through it. As per this analysis in the nutrient solution of the various samples indicate the proper nutritional balance for the crop is present in the soil. (Yinglu *et al*., 2007).

Heavy metal presence in agricultural soils

Liberation of heavy metal in the environment without proper treatment may increase the concentration of this in the soil. From our results it indicates that the plant cultured in land having significant level of heavy metals and this may affect the human normal life. Previous survey (GDPEMC 1990) indicated that the heavy metal background values of natural soil in Guangzhou were Cd (0.14 mg·kg−1), Cu (13.6 mg·kg−1) Ni (22.03 mg·kg−1), Pb (42.88 mg·kg−1) and Zn (58.1 mg·kg−1). Similarly in our studies suggest that our study area are contaminated with Nickel, zinc and cadmium. (Zhang *et al*., 2007).

Microbial biomass in heavy metal contaminated soil

Microbe plays an vital role in not only in the nutrient recycling process and also it simplify certain hard and complex materials to plant. Thus, microbe improves the soil quality by increasing the nutrients and also some of them provide security with it secondary metabolites. (Diels *et al*., 1999).

If the content of the heavy metal in the soil increases automatically the diversity of the microbes will affect. The level of heavy metal increased the content of the microbes also decreased. In this study the concentration of the heavy metals are inversely proposed. Heavy metals get accumulated in time in soils and

plants and could have a negative influence on physiological activities of plants (e.g. photosynthesis, gaseous exchange, and nutrient absorption), determining the reductions in plant growth, dry matter accumulation and yield (Devkota and Schmidt, 2000; Baker, 1981). In small concentrations, the traces of the heavy metals in plants or animals are not toxic (De Vries *et al*., 2007). Lead, cadmium and mercury are exceptions; they are toxic even in very low concentrations (Galas Gorchev, 1991).

Table 2. Agricultural soil analysis for Heavy metal (Nickel, Zinc and cadmium) using AAS analysis

Soil sample	Heavy metals availability	Concentration (ppm)
CS1	Nickel	3.9
	Zinc	2.5
	Cadmium	4.1
CS2	Nickel	2.6
	Zinc	3.2
	Cadmium	3.9
CS3	Nickel	2.7
	Zinc	4.1
	Cadmium	3.3
CS4	Nickel	2.9
	Zinc	3.2
	Cadmium	3.7
CS5	Nickel	3.4
	Zinc	2.8
	Cadmium	3.7

Values are represented as ppm concentration of heavy metal (Mixture of five random samples from single site).

Table -3. Estimation of microbial biomass from agricultural and contaminated agricultural soils

Soil Characters	**Sample**	**CFU**
Rhizosphere soil (NC)	NCS1	$26x10^8$
	NCS2	$3x10^7$
	NCS3	$34x10^6$
	NCS4	$28x10^7$
	NCS5	$11x10^8$
Rhizosphere soil (Cont)	CS1	$15x10^5$
	CS2	$3x10^4$
	CS3	$11x10^3$
	CS4	$9x10^4$
	CS5	$18x10^3$

NC – non contaminated soil; Cont – contaminated soil; CFU – colony forming unit

Reference

Abou, R., Shanab, A., Ghanem, K and Kalaibe, A. D. (2008).The role of bacteria on heavy metal by plants growing on multi – metal contaminated soils, *world J. Microbial, Biotechnol,* 24: 253-262.

Baker, A. J. M. (1981). Accumulator and excluders: Strategies in response of plant to heavy metals. *J. Plant Nutr,* 3: 643–654.

Cheng, S., Grosse, W., Karrenbrock, F and Thoennessen, M. (2003). Efficiency of constructed wetlands in decontamination of water polluted by heavy metals, *Ecol Eng* ,18: 317–325.

Diels,L., De smet, M., Hooyberghs,L and Corbisier, P. 1999. Heavy metals bioremediation of soil, *J.molecul.biotchnol*, 149-158.

De Vries, W., Romkens, P. F., Schutze, G. 2007. Critical soil concentrations of cadmium, lead, and mercury in view of health effects on humans and animals, *Reviews of Environment Contam and Toxicol,* 191: 91–130. [Pub Med]

Devkota, B., Schmidt, G.H. 2000. Accumulation of heavy metals in food plants and grasshoppers from the Taigetos Mountains, Greece, *Agricul, Ecosyst and Environment,* 78(1): 85–91.

Galas-Gorchev, H. 1991. Dietary Intake of Pesticide Residues: Cadmium, Mercury and Lead, *Food Add Cont.* 8: 793–806.

McGrath, S.P., Zhao, F.J., Lombi, E. 1995. Plant and rhizosphere process involved in phytoremediation of metal-contaminated soils. *Plant Soil*, 232(1/2): 207-214.

Patrick. L. 2003.Toxic metals and antioxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity.Altern Med Rev, *Environ Contam Taxicol,* May; 8(2): 106-28: Review.

Phipps, D. A. 1981. "Chemistry and biochemistry of trace metals in biological systems", in Effect of Heavy Metal Pollution on Plants, N. W. Lepp (Ed.), *App. Sci. Publishers.1-6.*

Sinha, S., Rai, U. N., Tripathi, R. D., Chandra, P. 1993. Chromium and manganese uptake by Hydrilla verticillata (I.f.) Royle : amelioration of chromium toxicity by manganese, *J. Environ Sci Health,* Part A 28(7): 1545–1552.

Slobodkin, 2005. Thermophilic microbial metal Reduction. *J. of Microbiol*, 74 (5): 501-514.

Vajpayee, P., Rai, U. N., Tripathi, R. D., Sinha, S., Chandra, P. 1995.Bioremediation of tannery effluent by aquatic macrophytes, *Bull Environ Contam Toxicol,* 55: 546–553.

Weis, J. S., Weis, P. 2004. Metal uptake, transport and release by wetlands plants

implication for Phytoremediation and restoration. *Environ Int.* 30(5): 739–753.

Yinglu – Feng zhu-jie chen, Haihuaggan and Yenbiaogue, 2007. Chemical fractionation of heavy metals in urban soils of Guangzhau, China, *Environ monit Assess,*134: 429-439.

Zhang X-H., Liu, J., Huang H-T., Chen, J., Zhu Y-N and Wang D-Q. 2007. Chromium accumulation by the hyper accumulator plant *Leersia hexandra* . Swartz. Chemosphere 67: 1138–1143.

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Original Article

Fluoride Removal from water by Sorbing on Plant and Fungal Biomass

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Abstract

The capacity of microbial biomass to remove hazardous element from aqueous solution even after being killed has been well documented. Such material could be used to decontaminate waste water originating from industries involved mining, refining, nuclear fuel processing etc. The present study identified saw dust, *Aspergillus penicilloides* and *Mucor racemosus* as potential biomaterial for calcium and fluoride removal. Maximum Ca^{2+} sorption was observed in saw dust however *Aspergillus penicilloides* and *Mucor racemosus* provide themselves as the better agent for f^- removal after Ca^{2+} treatment. This proves that the cations on the surface of biomass may be used to removal anions that are not usually removed as the cell envelops carry negative charges or their surface. Biosorption can therefore provide a technical answer to control pollution due to permissible, illegal and accidental discharge and hence may become an essential implement for environmental protection.
***Keywords*:** Biomass, Biosorption, *Aspergillus penicilloides*, *Mucor racemosus*

Introduction

There has been an ever increasing global concern about the fluoride anion, an excess of which can inflict numerous toxic effects on many biological systems (Bhatnagar and Bhatnagar 2000; Pushink and Miller 1990; Tsezos and Bell 1989) .Microalgal biomass has been extensively studied as a biosorbent for various heavy metals (Holan, Volesby and Prasetyo 1993; Wilde and Benemann 1993) and hazardous substances (Liu and Wu 1993; Tsezos Bell 1989) however being negatively charged surface it cannot absorb sufficient anions. Phosphate supply could modify flocculation and adsorption behavior in growing *Corynebacterium glutamicum* (Buch, Mozes, Wandrey and Rouxhet 1998). Fluoride sorption using a technique to pretreat surface with Ca^{2+} (Bhatnagar, Bhatnagar and Jha 2002). Biosorption removal of fluoride if possible, shall become an alternative or adjunct method the conventional technology of fluoride removal. In view of this, in the proposed study, fungal biomass and saw dust were used to pretreat with cations and find if fluoride removal may be affected using this technique.

Materials and Methods

Aspergillus penicilloides and *Mucor racemosus* were grown in Asthana and Hawker's (AH) medium-A (Asthana and Hawker 1936). The pH of medium was adjusted to 7.0 $\pm$ 0.1 with 0.1N NaOH or 0.1N HCl. Aseptically 1 ml culture from the growing phase was inoculated in 20-25 ml of AH medium in 100 ml Erlenmeyer flask and incubated for 2-3 days at 34 $\pm$ 1°C temperature.

Asthana and Hawker (AH) medium-A (Asthana and Hawker 1936)

D Glucose	5.00g
KNO_3	3.50g
KH_2PO_4	1.75g
$MgSO_4\,7H_2O$	0.75g
Distilled Water	1000ml

Harvesting the biomass

The biomass was obtained by centrifugation of growing culture at 8,000 r.p.m for 15 minutes followed by washing with double distilled water, recentrifugation and finely drying at 60º C for 72 hours in an incubator. It was than crushed using mortar and pestle to obtain a uniform powder. culture tube, washed thoroughly in running tap water before transplanting into small polycups containing mixture of sterilized sand and garden soil and irrigated with 1/10 diluted liquid MS medium and covered with poly bags for in vitro hardening. After hardening in polycups they were subsequently transferred to 15 cm diameter pots containing, sand and compost (2:1:1) and maintained under mist irrigation. Then they were shifted for field planting. Experiments were

performed with a minimum of 15 replicates for initiation and were repeated at least twice.

Calcium biomass analysis

0.1 g of dried biomass or filtered wood powder was suspended in test tubes containing 10 ml of aqueous $CaCl_2$ solution in the concentration varying from 30 to 100 µg ml^{-1} and allowed to stand for 5 hours after putting it mixed for 5 minutes on rotor. Before analysis the samples were centrifuged at 5000 r.p.m. for 15 minutes and the supernatant was collected and to determine calcium using Systronics Flame Photometer 128.

Fluoride treatment

Fresh biomass (0.1g) was treated as above with calcium chloride concentration at which maximum biosorption was obtained in the previous experiment. It was again separated by centrifugation and dried as earlier. 0.1 g of the dried material was suspended in centrifuge tubes containing 0-50 µg ml^{-1} (viz. 0, 15, 25, 50 µg ml^{-1}) fluoride, for 5 hour. After the treatment cells were separated by centrifugation. Analysis of residual fluoride in the supernatant was estimated using SPADNS method (Eaton *et al*., 1995). *Systronics* 118 UV-VIS Spectrophotometer was used for the purpose.

Results and Discussion

Saw dust and dead and dried biomass of *Aspergillus penicilloides* and *Mucor racemosus* were treated with aqueous solution of calcium chloride in the range of 30-100 µg ml^{-1} for a period of 5 hours. The Saw dust and *Mucor racemosus* sorbed maximal calcium at 50 µg ml^{-1}.While *Aspergillus* sp. display maxima at 60 µg ml^{-1} (Table 1 & Fig.1, 2, 3)

The uptake increased linearly as a function of calcium concentration till this concentration in each of the treatment, but the fall after this stage did not show any pattern.

To determine the effect of pretreatment of biomass with Ca^{2+}, the saw dust and *Mucor racemosus* biomass were first exposed to 50 µg Ca^{2+} ml^{-1} for 5hours and *Aspergillus penicilloides* with 60 µg Ca^{2+} ml^{-1} for 5 hours. Following this the Ca^{2+} treated biomass and saw dust were kept in varying concentrations of fluoride (viz. 0, 15, 25, 50 µg F^- ml^{-1}). Biomass without any prior treatment was used as control (Table 2).

From a solution of 50 µg F^- ml^{-1}, *Aspergillus penicilloides* could remove as much as 14 µg F^- ml^{-1} while *Mucor racemosus* removed 6 µg F^- ml^{-1} only on the other hand saw dust could remove only 2.5 µg F^- ml^{-1}.

In case of controls which were not treated earlier with Ca^{2+} removal was nil in most cases except 15 µg F^- ml^{-1} for saw dust and 50 µg F^- ml^{-1} for *Aspergillus penicilloides* proving the hypothesis that Ca^{2+} biosorption before fluoride treatment enhanced the rate of anion removal. Earlier reported (Bhatnagar , Bhatnagar and Jha 2002) 10 mg F^- l^{-1} removed by cyanobacterium *Anabana fertilissima* and 15 mg F^- l^{-1} *Chlorococcum humicola*. In comparison to this the present study showed that fungal biomass might be a better option for F^- removal after Ca^{2+} sorption. The difference in the abilities of plant (saw dust) and fungal biomass may be explained on the basis of their chemistry.

The structural polysaccharide cellulose in the most abundant natural polymer found in the world. Found in cell wall of plants, cellulose is one of the principle component provide physical structure and strength. Cellulose with β-(1,4) glycosidic linkage can adopt a fully extended conformation with alternating 180^o flips of the glucose units. Ca^{2+} ions from a dimmer complex with cellulose by interacting with 6-OH (Hydroxyl) group of upper layer of cellulose and 6-OH (Hydroxyl) group of lower layer layer of cellulose. Surface attachment of Ca^{2+} remains free to interact with anions. Therefore cellulose of saw dist shows maximum biosorption of Ca^{2+} but low biosorption of fluoride comparing chitin structure of fungal biomass (Garret and Grisham 1995) .Both fungi show better fluoride biosorption than the saw dust.

Table 1 - Calcium biosorption by Saw dust and *Aspergillus penicilloides* and *Mucor racemosus*

Treatment (µg Ca^{2+} ml^{-1})	Control (µg Ca^{2+} ml^{-1})	Saw Dust		*Aspergillus penicilloides*		*Mucor racemosus*	
		Concentration (After 5 hour)	Removal (µg ml^{-1})	Concentration (After 5 hour)	Removal (µg ml^{-1})	Concentration (After 5 hour)	Removal (µg ml^{-1})
30	31.41	27.66	3.75	29.33	2.08	26.85	4.85
40	40.43	33.3	7.13	36.66	3.77	35.8	4.63
50	50.00	42.73	7.27	44.06	5.94	44.83	5.17
60	59.05	52.8	6.25	54.4	4.65	53.13	5.92
70	71.15	66.1	5.05	66.16	4.99	66.16	4.99
80	79.36	71.1	2.26	76.76	2.6	75.4	3.96
90	92.58	88.1	4.48	89.3	3.28	88.27	4.31
100	96.88	89.93	6.95	93.33	3.55	95.85	1.85

Each experiment was performed with 10 replicates and was repeated thrice.

Table -2: Fluoride removal by Saw dust and *Aspergillus penicilloides* and *Mucor raracemosus*

Fluoride concentration	15 µg F ml^{-1}			25 µg F ml^{-1}			50 µg F ml^{-1}		
	Saw dust	*A. penicilloides*	*M. racemosus*	Saw dust	*A. penicilloides*	*M. racemosus*	Saw dust	*A. penicillo ides*	*M. racemos us*
Control (µg ml^{-1})	14.28	14.28	14.28	23.75	25	24	50	50	50
(Calcium Pretreated)	13.14	13.57	13.14	21.25	21	18.75	47.5	36	44
Removal (µg F ml^{-1})	1.14	0.71	1.14	2.5	4	5.25	2.5	14	6
(No Pretreatment)	13.14	14.28	14.28	23.75	25	24	50	46	50
Removal (µg F ml^{-1})	1.14	0	0	0	0	0	0	4	0

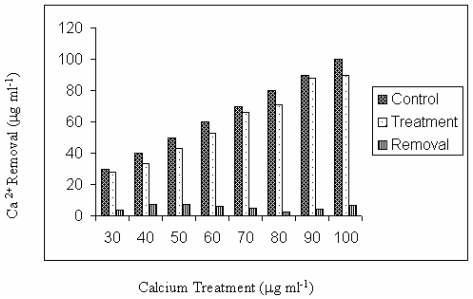

Fig.1: Calcium Removal by Saw dust

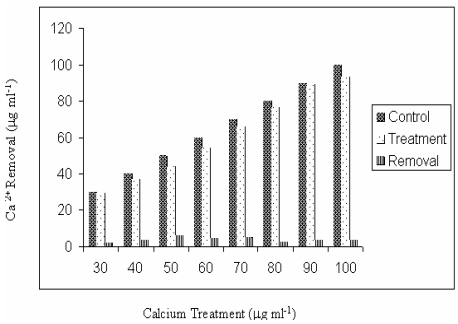

Fig.2: Calcium removal by *Aspergillus penicilloides* biomass

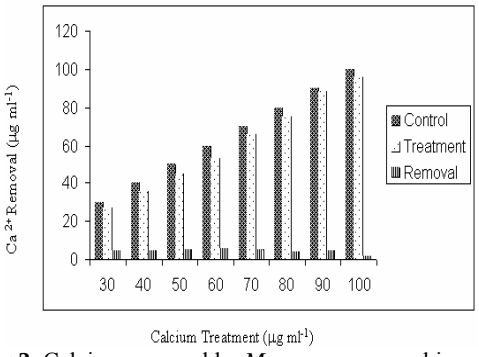

Fig.3: Calcium removal by *Mucor racemosus* biomass

Conclusion

The capacity of microbial biomass to remove hazardous element from aqueous solution even after being killed has been well documented. Such material could be used to decontaminate waste water originating from industries involved mining, refining, nuclear fuel processing etc.

The present study identified saw dust, *Aspergillus penicilloides* (Divsion-*Mycota*) and *Mucor racemosus* (Division-*Mycota*) as

potential biomaterial for calcium and fluoride removal.Maximum Ca^{2+} sorption was observed in saw dust however *Aspergillus penicilloides* and *Mucor racemosus* provide themselves as the better agent for f^- removal after Ca^{2+} treatment. This proves that the cations on the surface of biomass may be used to removal anions that are not usually removed as the cell envelops carry negative charges or their surface.Biosorption can therefore provide a technical answer to control pollution due to permissible, illegal and accidental discharge and hence may become an essential implement for environmental protection.

References

Asthana, R.D. and Hawker, L.E. 1936. The influence of certain fungi on the sporulation of *Milanlspora desiruens* shear and of some other Ascomycetes. *Ann botany (handon*)., 50: 325-344.

Bhatnagar, M., and Bhatnagar, A., 2000. Algal and cyanobacterial responses to fluoride. *Fluoride*., 33: 55-65.

Bhatnagar, M., Bhatnagar, A., and Jha, S., 2002. Interactive biosorption by microalgal biomass as a tool for fluoride removal. *Biotechnology Letters*., 24: 1079-1081.

Buchs, J., Mozes, N., Wandrey, C., Rouxhet, P.G. 1998. Cell adsorption control by culture conditions. *Appl. Microbiol. Biotechnol.*, 29: 119-128.

Eaton, A.D., Clesceri, L.S., and Greenberg, A.E., 1995. APHA, *Standard Methods for Examination of Water and Wastewater* (19/e). Washington D.C. Amercian Public Health association.

Garret, R.H., and Grisham, C.M., 1995. Carbohydrates and Glyco-Conjugates of the Cell Surface. In: *Biochemistry* 1/e , Saunders College publishers, New York, pp.334.

Holan, Z.R., Volesby, B., and Prasetyo, I.,1993. Biosorption of cadmium by biomass of marine algae. *Biotechnol. Bioeng*., 41: 819-825.

Liu, H.H., and Wu, J.T., 1993. Uptake and recovery of americium and uranium by *Anacystis* biomass. *J. Environ*., *Sci. Health.*, 28(2): 491-504.

Pushink, J.C., Miller, G.M., 1990. The influences of elevate environmental fluoride on the physiology and metabolism of higher plants. *Fluoride*., 23: 5-19.

Treshow, M., and Anderson, F.K., 1989. *Fluoride: Origins and Effects*. New York: John Wiley and Sons., pp.61-76.

Tsezos, M., and Bell, J.P., 1989. Comparsion of biosorption and desorption of hazardous organic pollutants by live and dead biomass. *Water Res*., 23: 561-568.

Wilde, E.W., Benemann J.R., 1993. Bioremoval of heavy metals by the use of microalgal. *Biotech. Adv*., 11: 781-812.

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Original Article

Metal concentration in Manakudy estuarine sediments South West Coast of India

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Abstract

Estuary is a transition zone between land and sea as well as freshwater and saltwater. Sediments are important sinks of various pollutants and play a significant role in the remobilization of contaminants in aquatic system between water and sediments. Low content of organic carbon is attributed to low bioproductivity and active hydrodynamics. The Electrical conductivity is continuously decrease from station I to V. Among the trace metals copper is very low. This investigation was under taken to study the metal concentration in Manakudy estuarine sediments.
Keywords: Sediments, Trace metals, Estuary, Major elements

Introduction

The chemistry and ecology of an estuarine system are entirely different from the fluvial as well as the marine system. Estuarine environment is characterized by a constantly changing mixture of salt and freshwater. Sedimentary material carried out into the estuary from the sea and rivers, which form the mud flats. Estuaries have been claimed to be the most productive natural habitats in the world. They provide vital spawning nursery and feeding grounds for fish and shellfish and support many species including wading birds, reptiles, migratory water fowl, shore birds, amphibians and mammals.

Many geochemical studies have been carried out on the shelf and slope sediments of the south west coast of India (Shajan kuttickat, 2000).Geochemical studies of bottom sediments of water bodies like rivers, estuaries and marine basins are helpful in understanding the different sediment sources and element distribution pattern Such studies throw light on establishments of sediment characteristics, facies relationship and depositional process (Gandhi *et al.*, 2000).

The heavy minerals in the shelf are being supplied by the rivers and from the lowered sea level. Apart from this, the daily rise and fall of the tides, and the movement of saltwater, influence the characteristics and composition of the sediments (Prakash, 2000).

Rates of sedimentation vary from as low as 1mm yr^{-1} in coastal marine water to 10-20 mm yr^{-1} in some riverine and estuarine systems. Accelerated sedimentation is generally detrimental to the estuarine health while geological rates of sedimentation are usually beneficial (Rengaswamy *et al.*,2005). Rivers, the major source of irrigation are used as repositories for disposal of domestic sewage, industrial effluents containing toxic substances, heavy metals and agricultural run-off. The amount of fresh water flowing into an estuary varies from season to season and from year to year. Moreover, in the estuary the water carrying agricultural, industrial and domestic wastes are deposited as sediment (Daskalakis and O' connor, 1995).

Estuaries are often contaminated with a range of organic and inorganic contaminants. Sources of environmental contaminants to the coastal system are numerous. They enter the estuarine system through different pathways mainly rivers. Contaminated sediments may be directly toxic to aquatic life or through bio accumulation. The biomagnification can cause long term chronic effects (Swartz *et al.*, 1985).

Contamination of sediments, water resources and biota by heavy metals are of major concern. Many industrialized areas are affected due to their toxicity, persistence. and bio-accumulative nature. Organic carbon in riverine and estuarine sediments is controlled mostly by

the rate of organic to inorganic constituents, primary productivity, composition and texture of the sediments. Textural control over total organic carbon is indicated by the correlation of total organic carbon with sand, silt and clay percentages of the sediments. Association of total organic carbon with clay minerals is of particular significance in estuarine sediments(Muraleedharan Nair *et al.*,2002).

Plant nutrients like calcium, magnesium potassium, sodium and phosphorus are present in the minerals and in solution. Oxygen, silicon and aluminum occur as constituents of minerals and as oxides. Nitrogen and phosphorus are present in organic and inorganic forms. Metals may be present in the estuarine system as dissolved species, free ions or forming organic complexes with humic and fulvic acids. The metals present in the environment cannot be degraded either by chemical or biological process.

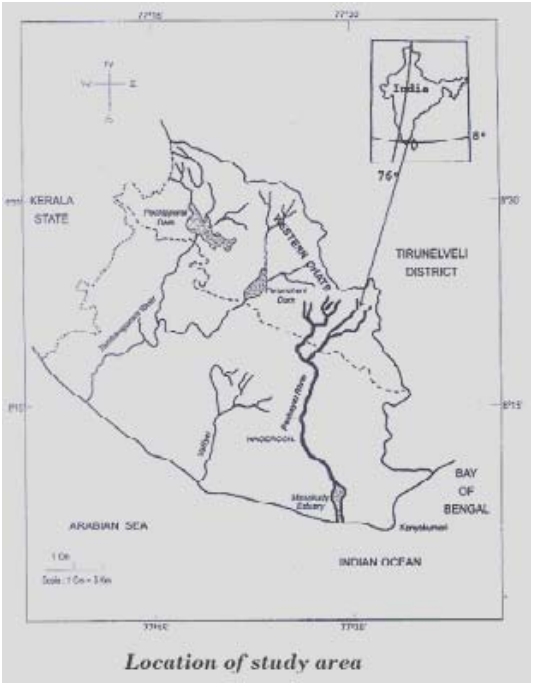
Many of the trace metals are highly toxic to humans and other living organisms. They may be bioconcentrated in the food chain. Estuarine and coastal sediments act as ultimate sink for trace elements that are discharged into the aquatic environment (Achyuthan *et al.*,2002). Physical properties such as grain size and density are important parameters in sedimentation and transport processes. Therefore, the present study has been under taken to investigate the metal concentration in Manakudy estuary.

Materials and Methods

Study Area

Manakudy estuary is the second largest estuary in Kanyakumari District. It has a total area of 145 hectares. The tail end of pazhayar river merges with the Arabian sea at Manakudy. Manakudy estuary is situated about 8 kilometers north west of cape comorin falling with in the latitude $8^{0}4^{1}$ and $8^{0}21^{1}$N and longitude $77^{0}26^{1}$ and $77^{0}30^{1}$E. The climate of the region is greatly influenced by both the South -West and North- East monsoons. There is not much variation in the mean monthly air temperature (36.67^{o}C to 23.89 ^{o}C). Shallow open waters, fresh and small marshes, sandy beaches, muddy flats, rocky shores, oyster reefs, mangrove forests, river deltas, tidalpools and sea grass are the some important habitats found in and around the estuary. Khondalites, charnockite, and the river and marine aluviam are the geological formations observed in these study area.Water samples were collected from five different stations from estuarine mouth bed to river basin. A total of twenty five sediment samples were collected in polythene bags labelled before transporting to the laboratory.

The collected samples were initially air



Location of study area

dried and finely powdered using agate motor. The percentage of sand silt and clay were determined by the pipette method (Krumbein and pettijohn, 1936). Percentage of organic carbon was determined by titration method of Walkley Black (1934) as well as Elwakeel and Riley (1957). Nitrogen was estimated by kjeldhal method (Technicon Industrial System,1973). Electrical conductivity was measured using Elico conductivity bridge and the pH was recorded using Elico pH meter. Silicondioxide was estimated gravimetrically (AOAC,1962) Calcium oxide and magnesium oxide were estimated titrimetrically by EDTA method (APHA,1998). Phosphorus was estimated by ammonium phosphomolybdate method (APHA,1998). Aluminum was estimated by Alizarin Red-5 method. Iron was estimated by 1.10- phenanthroline method. Sodium oxide and potassium oxide were estimated by flame photometric method (APHA, 1998). All the trace

metals were estimated by Atomic Absorption Spectroscopic method.

Results and Discussion

The Present study on the metal concentration of the sediments in Manakudy estuary in twenty five samples spread over all the five stations indicate wide variation for all the parameters studied. The values are tabulated and represented graphically and pictorially.

From the results, the low C/N ratio (3.3) may be due to lack of suspended matter in the estuary. The higher C/N ratio (10.7) is due to terrigenous organic matter enriched with residual of mangrove grasses which contain high contents of lignin and cellulose. Higher concentration of phosphorus is due to the larger supply of terrigenous material by the river, organic productivity and agricultural waste discharge from the paddy fields of the region. Low concentration of phosphorus is due to the process of flocculation and the change in salinity. low in station I due to low quantity of fine sediments and high degree of desorption under saline condition.

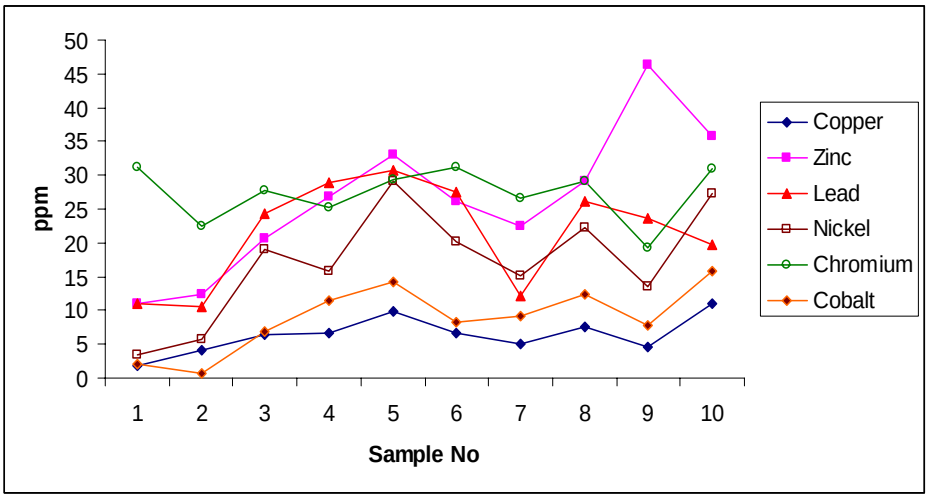


Fig-1: Variation of Trace Metals in sediments in ppm units

Accordingly, Station II and III are more polluted with respect to trace elements especially zinc, lead and chromium. The concentration of nickel is also higher in these region, however it is associated more in the coarser sediments rather than in fines. The higher concentration of lead in sediments is indicated by the low degree of desorption of the metal under estuarine conditions. Based on the analytical works, the mouth of the estuary has very low quantity of fine sediments due to turbulent conditions. The continuous decrease of electrical conductivity from sea mouth towards the river mouth is an indication of calm hydrodynamic conditions prevailing in the estuary, as the sediments have not undergone sufficient mixing.

The organic carbon is highly positively correlated with nitrogen (r =0.95) revealing that the accumulated fine sediments are nitrogenous organic matter The linear Fe-Al relationship indicates that reactive iron is retained more efficiently in fine sediments due their lower permeability of tidal water flushing.

Strong positive correlation between copper and nickel (r =0.94) indicates the strong association between them, because both are having same ionic radii in + 2 state (0.72^0 A). Nickel is positively correlated with lead (r= 0.74) and are mutually correlated to organic carbon (Ni : r =0.51, and pb: r =0.49)due to high degree of adsorption of these metal ions and their complexing ability with organic carbon.

Table - 1: Physico-Chemical properties of the sediments

Locations	pH %	EC dS/m	Organic Carbon%	Nitrogen ppm	Phosphorus ppm	C/N ratio
1	8.82	4.94	0.18	280	250	6.428
2	8.68	7.93	0.24	280	250	8.571
3	8.70	5.68	0.29	420	300	3.320
4	8.75	5.4	0.29	420	340	3.320
5	8.64	3.18	0.30	420	340	7.142
6	8.55	2.8	0.36	420	300	8.571
7	8.49	2.61	0.36	420	340	9.047
8	8.36	1.58	0.41	420	300	8.571
9	8.28	2.24	0.41	560	380	9.761
10	8.05	2.32	0.57	840	430	9.464
11	7.83	2.91	0.57	840	430	10.178
12	8.31	3.71	0.68	700	430	8.095
13	7.98	3.58	0.69	840	380	9.857
14	8.04	2.20	0.71	840	340	8.452
15	8.12	3.79	0.72	700	300	8.571
16	7.72	2.74	0.60	700	380	8.571
17	8.27	2.46	0.53	560	430	8.142
18	7.85	2.58	0.51	560	470	7.571
19	7.43	2.08	0.41	420	510	9.107
20	7.62	1.73	0.36	420	470	7.321
21	7.55	1.34	0.38	520	510	8.571
22	7.45	1.22	0.45	520	430	9.047
23	7.35	1.18	0.45	280	430	8.653
24	7.46	1.17	0.30	280	340	10.714
25	7.25	1.35	0.32	420	300	7.619

Table – 2: Percentage of Major elements

Locations	Si	K	Ca	Mg	Na	Fe	Al
1	41.0	0.049	4.132	0.9	0.111	0.28	0.832
2	39.8	0.057	4.054	0.84	0.103	0.84	1.716
3	39.6	0.057	0.795	0.66	0.066	2.17	3.328
4	39.6	0.082	0.752	0.3	0.066	0.98	3.952
5	40.7	0.082	2.186	0.372	0.074	0.63	1.612
6	40.5	0.090	1.434	0.408	0.066	0.56	2.184
7	40.7	0.098	0.795	0.432	0.074	0.77	1.768
8	40.4	0.098	0.752	0.42	0.066	0.84	2.496
9	40.9	0.098	0.397	0.192	0.066	0.77	2.808
10	40	0.131	0.355	0.18	0.074	1.05	3.328
11	39.1	0.164	0.319	0.24	0.103	1.19	3.588
12	41.9	0.098	0.553	0.06	0.066	0.98	2.392
13	41.1	0.098	0.198	0.156	0.074	0.91	2.184
14	39.3	0.172	1.065	0.132	0.074	1.75	4.16
15	41.4	0.114	0.873	0.216	0.066	0.77	2.548
16	40.9	0.094	0.795	0.252	0.103	0.91	3.224
17	38.8	0.106	0.397	0.3	0.066	1.68	4.212
18	38.5	0.098	0.276	0.192	0.066	1.82	4.888
19	38.4	0.090	0.241	0.204	0.074	1.61	5.564
20	37.6	0.090	0.198	0.168	0.066	1.82	5.668
21	37.3	0.098	0.319	0.24	0.074	1.89	6.136
22	39.1	0.098	0.113	0.084	0.081	1.68	4.212
23	39.6	0.147	0.120	0.078	0.081	1.19	4.316
24	38.8	0.155	0.156	0.06	0.118	1.47	4.628
25	38	0.082	0.156	0.066	0.111	1.68	4.992

Table – 3: Concentration of trace elements in ppm units

Samples	Cu	Zn	Pb	Ni	Cr	Co
I	1.775	11.09	10.92	3.35	31.1	2.02
II	4.175	12.38	10.47	5.65	22.37	0.75
III	6.325	20.73	24.22	18.95	27.75	6.775
IV	6.7	26.79	28.8	15.77	25.3	11.55
V	9.8	33.08	30.82	29.02	29.42	19.25
VI	6.6	26.1	27.5	20.22	31.12	8.3
VII	5.05	22.45	12.27	15.15	26.55	9.1
VIII	7.475	29.13	26.2	22.17	29.15	12.3
IX	4.525	46.22	23.62	13.57	19.22	7.9
X	11.02	35.74	19.72	27.22	30.92	15.72

Reference

Achyuthan, H., Richardmohan, D., Srinivasalu, S. and Selvaraj, K. 2002. Trace metal concentration in the sediment cores of estuary and tidal zones between Chennai and pondicherry, along the east coast of India. *Ind. J. Mar. Sci*. 141-149.

American public health Association (APHA),1998. Standard methods for the Examination of waters and wastewater, Washington, DC, USA, edn. 20.

Daskalakis, K.D. and O' connor. T.P.1995. Distribution of chemical concentration in US Coastal and estuarine sediment. *Mar. Environ. Res*, 40: 381-399.

Elwakeel,S.K.and Riley,J.P.1957. Determination of organic carbon in marine muds. *J.Cons. Per. Intl. Expl. Mer*, 22:180-183.

Gandhi, M.S., Seetharamaiah, J. and Murthy, P.B.2000. Sediment characteristics in the deposistional environments of the Krishna delta, east coast of india *Ind. J. Mar. Sci.* 29. 128.132.

Krumbein, W.C and Pettijohn. F.J.1938. Manual of sedimentary petrography. *Appleton centuary Co., Inc., Newyork, USA* 1-549.

Muraleedharan Nair, M.N. and Ramachandran, K.K.2002. *Ind. J. Mar. Sci.* 31 (4): 295-304.

Prakash, T.N.2000. Sediment distribution and placer mineral enrichment in the inner self of quilon SW coast of India. *Ind. J. Mar. Sci* 29: 20-127.

Rengasamy Alagarsamy and Jingzhang,2005. Comparative studies on trace metal geochemistry in Indian and chineese rivers. *Current Science*, 89: 25.

Shajan Kuttickat Paul.,2001. Geochemistry of bottom sediments from a river – estuary shelf mixing zone on tropical south west coast of India. *Bull. Geol. Surv. Japan.* 2. 1 and 2.

Swartz, R., Deben, W., Jones, J., Lamberson , J. and Cole, F.1985. Phoxocephalid amphipod bioassay for marine sediment toxicity. Aquatic Toxicology and Hazard Assessment. *In Seventh Symposium*,R.Cardwell.

Purdy, R. and Banner, R.1985. eds. pp.284-307 ASTM,STP 854.

Technicon Industrial Systems. Industrial methods for Technicon, auto analyzer, Tarrytown, Newyork (1973).

Walkley, A. and Black, I.A.1934. An examination of the Dethreff method for determining soil organic matter and a proposed modification of the chromic acid titration method *Soil.Sci.* 27:29-38.

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Original Article

Intra specific hybridization between *Amhiprion sebae* and *A. polymnus* under captive conditions

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Abstract

The hybridization experiment was carried between two different species (Amhiprion sebae and A. polymnus) belonging to the same family to find out the morphometric and meristic characters characters. After the breeding and hatching, the hybrids were generally intermediate between the parental species of the following characters like standard length, caudal peduncle length, pectoral to ventral fin origin/ventral to anal fin origin, dorsal fin base length, lateral line scales, scales above lateral line, scales below lateral line etc. Finally the microscopic examination disclosed that the hybrids were neuter.

Keywords: *Amphiprion sebae; A. polymnus;* interspecific hybridization; sea anemone; intraspecific hybridization

Introduction

Inter and intra specific hybridization has been considered as one of the important mechanisms of obtaining better hybrids in the ornamental fish culture. Hybridization has been broadly defined as interbreeding between genetically distinct populations (Arnold, 1997) and hybrid zones are geographic areas where genetically distinct populations come into contact and hybridize.

Hybridization between individuals from genetically distinct populations is gained considerable importance in genetic improvement of fish species. Hybrid zones, regions where from genetically distinct populations interbreed to form genetically mixed offspring, have been recognized as fertile grounds for evolutionary studies concerning models of speciation, selection, recombination, the maintenance of species boundaries, and the evolution of host-parasite interactions (Boecklen and Spellenberg, 1990 and Harrison, 1990). In conservation biology and resource management, hybridization between endemic species and introduced species or between wild and cultured populations (Elo *et al.,* 1997; Jansson and Oest, 1997) is a topic of great concern.

Many investigators have carried out hybridization experiments with salmonid, cyprinid and gasterosteid fishes. With cyprinid fishes, Suzuki (1966 and 1968) performed experimental hybridization among several species. However, previously reported studies dealt almost exclusively with survival, viability and morphological analyses and with only a few species. In the present study, the hybridization experiment was carried out between the anemone fish species, *Amphiprion sebae* and *A. polymnus.*

Materials and Methods

The anemone fish, *Amphiprion sebae* (Bleeker) and *A. polymnus* were collected from Gulf of Mannar region with the help of SCUBA divers at depths of 4 to 5 meters along with their host anemones (*Stichodactylus haddoni*) (Plate-1: 1&2). They were transported to the laboratory in plastics buckets fitted with battery aerators. They were fed *ad libitum* with a mixed diet consisting of clam meat, *Artemia*, polychaete worm, minced beef etc. Methods for hybridization were adopted from Shen (1998). The meristic and morphometric characters were followed by Kasama and Kobayasi (1990).

Two breeding pairs such as *Amphiprion sebae* ♀ and *A. polymnus* ♂ and *A. sebae* ♂ and *A. polymnus* ♀ were used.

Results

Development and viability (A. sebae ♀ and A. polymnus ♂)

About 57.3% of 2,275 eggs produced from three pairs of hybrid brood fishes between *A. sebae* ♀ and *A. polymnus* ♂ developed upto gastrular stage. In this stage, we have recorded high mortality and almost all surviving embryos hatched out between 6 to 8 days after fertilization. About 41.6% of the eggs alone produced larvae, among these more than 30% of the larvae suffered from diseases like edema, bent bodies and circulatory disorders and they have died after 2 to 5 days of hatching. Among the surviving larvae seemed to develop normally, but many could not feed and died within 10 days after hatching. Only about 52 hybrids were raised up to the young stage but most of the larvae did not feed properly and died due to malnutrition caused by digestive disorders. Hybrids reared up to the preadult like stage were only 14.

Development and viability (A. sebae ♂ and A. polymnus ♀)

About 52.6% of 1,963 eggs produced from three pairs of hybrid brood fishes between *A. sebae* ♂and *A. polymnus* ♀ developed up to gastrular stage. After fertilization, the embryo mortality was high and the eggs were hatched out between 6 to 8 days and about 39.7% of the eggs were produced larvae. In this experiment also more number of larvae was died with some sort of physiological problems and digestive disorders. After hatching, the remaining larvae developed normally, but most of them could not feed properly and died within 10 days. Only 12 hybrids reached pre adult stage.

Morphometric and meristic characters

The hybrids were distinguished into two types on the basis of 16 morphometerc and meristic characters. The data on the morphometric amd meristic characters of *A. sebae, A. polymnus* and their hybrids were presented in Table 1,2 and 3.

Morphometric and meristic characters of A. sebae

The *A. sebae* was similar in appearance to *A. polymnus* but had a pale caudal peduncle. The mid-body bar of the fish, unlike its close cousin was almost complete at the belly. The body was usually high (generally terete in the plankton pickers) and compressed. The colour of the *A. sebae* was brownish black, with two milky white cross bands, the anterior proceeded from over the nape to the sub-opercle, touching the orbit anteriorly and covering most of the opercle posteriorly. The second band proceeded from the three last dorsal spines and four first rays ending anteriorly a little in front of the vent and the end of the free portion of the tail and caudal fin canary yellow (Table 1 and Plate 1: 5 & 6).

Mouth was small and the length of the lower jaw was 0.70 cm. Pre-orbital and post orbitals were with one or more serrations and the length was about 0.90 and 1.05 cm respectively. The inter-orbital width was 3.00cm and length of orbit was 0.75cm. The number of dorsal fin rays, pectoral fin rays, pelvic fin rays and caudal fin rays were 26, 8, 16 and 21 respectively. The head length, width and body depth about 2.3, 1.65 and 3.75 cm respectively. The length of the caudal peduncle, pre –dorsal length and pre-anal length were 1.45, 2.8 and 4.75 cm respectively. Lateral line was interrupted after about 38 scales (Table 1).

Morphometric and meristic characters of A. polymnus

The colour of the *A. polymnus* was dark brown with broad white bar just behind the eye on middle of back an abbreviated white saddle or on middle of the side a partial to complete white bar that slanted slightly backwards, extending onto middle and rear parts of dorsal fin. The caudal fin was mainly dark brown with broad whitish margins, the dark part tapering in width posteriorly, breast and belly either yellow orange or dark brown (Table 2 and Plate 1: 4).

The number of dorsal fin, pectoral fin, pelvic fin and caudal fin rays were 25, 7, 17 and 18 respectively. The head length, width and body depth were about 1.95, 1.70 and 3.35 cm respectively. Mouth was small and the length of the lower jaw was 0.55 cm. Pre-orbital and post orbitals were with one or more serrations and the length about 0.65 and 0.90 cm respectively. Lateral line was interrupted

after about 36 – 40 scales. The inter-orbital width was 2.40cm and length of orbit was 0.70cm. The length of the caudal peduncle, pre –dorsal length and pre-anal length were 1.45, 2.3 and 4.10 cm respectively (Table 2).

Morphometric and meristic characters of hybrid between A. sebae and A. polymnus

The hybrid colour was intermediate between their parent species. The colour of the hybrid was brownish black, with two milky white cross bands, the anterior proceeded from over the nape to the sub-opercle, touching the orbit anteriorly and covering most of the opercle posteriorly. The second band proceeded from the three last dorsal spines and four first rays ending anteriorly (like *A. polymnus*) (Plate: 1).

The *sebae*-like hybrids had the head length, width and body depth about 2.2, 1.70 and 3.70 cm respectively. Mouth was small and the length of the lower jaw was 0.65 cm. The number of dorsal fin, pectoral fin, pelvic fin and caudal fin rays were 26, 7, 16, 20 respectively. The length of the caudal peduncle, pre –dorsal length and pre-anal length were 1.45, 2.7 and 4.70 cms respectively. Pre-orbital and post orbitals were with one or more serrations and the length was about 0.75 and 1.00 cm respectively. Lateral line was interrupted after about 38 – 40 scales. The inter-orbital width was 2.90cm and length of orbit was 0.75cm. The dorsal and anal fin bases were about 2.45, 1.20cm respectively. The length of the dorsal spine, anal spine, dorsal ray and anal ray about 0.45, 1.10, 0.55 and 0.70 cms respectively (Table 3 and Plate 1: 5 & 6).

Table-1: Morphometric and meristic characters of *A. sebae*

Characters	***A. sebae***
Total length (cm)	10.5
Standard length (cm)	8.3
Dorsal fin rays	26
Pectoral fin	8
Pelvic fin	16
Caudal fin	21
Head length (cm)	2.3
Head depth (cm)	1.65
Body depth (cm)	3.75
Length of upper jaw	0.70
Pre anal length (cm)	4.75
Snout length (pre orbital) (cm)	0.80
Post orbital (cm)	1.05
Inter-orbital width (cm)	3.00
Length of orbit (cm)	0.75
Caudal peduncle length (cm)	1.45
Pre dorsal length (cm)	2.8

Table-2: Morphometric and meristic characters of *A. polymnus*

Characters	***A. polymnus***
Total length (cm)	8.85
Standard length (cm)	6.8
Dorsal fin rays	25
Pectoral fin	7
Pelvic fin	17
Caudal fin	18
Head length (cm)	1.95
Head depth (cm)	1.7
Body depth (cm)	3.35
Length of upper jaw	0.55
Pre anal length (cm)	4.1
Snout length (pre orbital) (cm)	0.65
Post orbital (cm)	0.9
Inter-orbital width (cm)	2.40
Length of orbit (cm)	0.70
Caudal peduncle length (cm)	1.45
Pre dorsal length (cm)	2.30

The *A. polymnus*-like hybrids, mouth was small and the length of the lower jaw was 0.60 cm. Pre-orbital and post orbitals were with one or more serrations and the length of about 0.60 and 0.85 cm respectively. The inter-

orbital width was 2.50cm and length of orbit was 0.70cm. The number of dorsal fin, pectoral fin, pelvic fin and caudal fin rays were 25, 6, 18 and 19 respectively. The head length, width and body depth were about 1.90, 1.75 and 3.45 cm respectively.

The length of the caudal peduncle, pre – dorsal length and pre-anal length were 1.40, 2.7 and 4.15 cm respectively. The dorsal and anal fin base was about 2.25, 0.80cm respectively. The length of the dorsal spine, anal spine, dorsal ray and anal ray were about 0.45, 1.20, 0.60 and 0.70 cms respectively. Lateral line was interrupted after about 36 – 40 scales (Table 3 and Plate 1: 4).

Discussion

Fish hybridization was favoured by genetic factors such as compatibility of genes (Dubois, 1981), reproductive behaviour (Pepin *et al.,* 1970) and ecological factors such as coincidence of reproduction (Hubbs, 1955). In nature, hybridization was usually not a very extensive or massive phenomenon. Under natural conditions genetic incompatibility produced various blocks affecting the viability and fertility of hybrids (Hubbs, 1961), so that hybrids were often degenerate and parental populations could not be maintained. Hybridization could be increased in some cases, such as by the introduction of a species into an aquatic system where closely related species live (Daget and Moreau,1981) or where the environment was changed either through natural causes or by human influence (Hubbs, 1955). The massive hybridization between *Amphiprion sebae* and *A. polymnus* in the laboratory conditions may be attributed to the latter.

Table- 3: Comparison of morphometric and meristic characters of a hybrid of *A. sebae* and *A. polymnus*

Characters	*A. sebae*	Hybrids		*A. polymnus*
		A. sebae	*A.polymnus*	
Total length (cm)	10.5	5.2	5.4	8.85
Standard length (cm)	8.3	4.15	4.15	6.8
Dorsal fin rays	26	25	25	25
Pectoral fin	8	8	7	7
Pelvic fin	16	15	16	17
Caudal fin	21	20	18	18
Head length (cm)	2.3	1.30	1.1	1.95
Head depth (cm)	1.65	1.20	1.25	1.7
Body depth (cm)	3.75	1.85	1.75	3.35
Length of upper jaw	0.70	0.25	0.25	0.55
Pre anal length (cm)	4.75	2.45	2.4	4.1
Snout length (pre orbital) (cm)	0.80	0.25	0.25	0.65
Post orbital (cm)	1.05	0.70	0.70	0.9
Inter-orbital width (cm)	3.00	1.80	1.4	2.40
Length of orbit (cm)	0.75	0.45	0.4	0.70
Caudal peduncle length (cm)	1.45	0.65	0.55	1.45
Pre dorsal length (cm)	2.8	1.55	1.55	2.30

Shen (1998) reported the inter-specific hybridization between protogynous angelfish species (*Pomacanthus semicirculatus* and *P. imperator*). In his study, the caudal fin of *P. semicirculatus* was black colour with wave like markings and in the case of *P. imperator,* the caudal fin was yellow with blue colour. But the hybrids were more resembled with the parental species of *P. imperator*. In the present study, the *A. sebae* and *A. polymnus* hybrids showed a unique difference in their caudal fin. *A. sebae* like hybrids had yellow coloured caudal fin, *A. polymnus* like hybrids had brown coloured caudal fin with white margins and some hybrids showed mixed colouration in their caudal fin. The phenotype of the hybrids was distinctly distinguished into two types, with the hybrid index for these two types being not intermediate but closer to either *A. sebae*. Hence, the present study corroborated with the earlier findings. According to Shen (1998), the

morphometric and meristic characters like standard length, total length, dorsal, caudal, pelvic, pectoral fin rays, head length and depth, body depth, length of caudal peduncle, pre-dorsal, pre-anal length etc of the protogynous angel fish hybrid were more or less similar to that of their parent species. The present findings also correlated with the earlier findings.

Suzuki (1968) reported that interspecific hybrids between *Carasius auratus auratus* (Linnaeus) female and *Gnathopogon elongatus alongatus* male were similar to *C. auratus auratus*, because *C. auratus auratus* was the native species. In the present study (*A. sebae* ♂ and *A. polymnus* ♀ and *A. sebae* ♀ and *A. polymnus* ♂), the hybrids resembled more with the parental species *A. sebae*, because which was originated from Indian Ocean, but in the case of *A. polymnus* originated from Pacific Ocean. So the present findings also correlated with the previous study of Suzuki.

According to Shen (1998), the presence of eye band was illogical in the hybrid (butterfly fish) between *Chaetodon guentheri* and *C. nippon* combination, since an eye band was absent on both species. The present findings were also agreed with these findings. Finally the microanatomical examination disclosed that the hybrids were neuter.

References

Arnold, M., 1997. Natural hybridization and evolution. Oxford University Press, Oxford.

Boecklen, W.J. and Spellnberg, R., 1990. Structure of herbivore communities in two oak (*Quercus* spp.) hybrid zones. *Oecologia*, 85: 92 – 100.

Daget, J. and Moreau, J., 1981. Hybridization introgressive entre duex especes de *Sarotherodon* (Pisces, Cichlidae) dans un lac de Madagascar. Bull. Mus. Natn. Hist. nat. Paris. *Zool.Biol.Ecol.,* 3: 689 – 703.

Dubois, A., 1981. Quelques reflexions sur la notion de genre Zoologie. *Bull.Soc.Zool. Fr.,* 106: 503 – 513.

Elo, K., Ivanoff, S., Vuorinen, J.A. and Piironen, J., 1997. Inheritance of RAPD markers and detection of interspecific hybridizations with brown trout and Atlantic salmon, *Aquaculture,* 152: 55 – 65.

Harrison, R.G., 1990. Hybrid zones: windows on the evolutionary process. *Otf. Surv. Evol. Biol.*, 7: 69 – 128.

Hubbs, C.C., 1955. Hybridization between fish species in nature. *Systematic Zoology.* 4: 1 – 20.

Hubbs, C.C., 1961. Isolating mechanisms in the speciation of fishes. Univ.Texas Symposium on Vertebrate Speciation, pp. 5 – 23.

Jansson, H. and Oest, T., 1997. Hybridization between Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in a restored section of the river Dalaelven, Sweden. *Can.J. Fish. Aquat. Sci.,* 54: 2033 – 2039.

Kasama, M. and Kobayasi, H., 1990. Hybridization experiment between *Carassius carassius* (female) and *Gnathopogon elongatus elongatus* (male). Hybridization experiments in Cyprinida (V). *Jap.J.Ichthyol.*, 36(4): 419-426.

Pepin, H., Moreau, G., Marazzato, S. and Grey, J., 1970. Biometrie d'un hybride naturel de poisons Cyprinidae, la Breme de Buggenhagen, Ann. *Hydrobiol.,* 1(1): 43 – 54.

Shen, 1998. The first record of a hybrid, *Pomacanthus semicirculatus X P. imperator,* from Nan-Wang, Southern Taiwan. *Tropical Fish Hobbyist.,* 26 – 28.

Suzuki, R., 1966. Hybridization experiments in cyprinid fishes X *Gnathopogon japonicus* (male) X *Pseudogobio esocinus* (female). *Bull. Freshw.Fish.Res.Lab.,* 16(1): 65 – 69.

Suzuki, R., 1968. Hybridization experiments in cyprinid fishes XI. Survival rate of F_1 hybrids with special reference to the closeness of taxonomical position of combined fishes. *Bull. Freshw.Fish.*

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Original Article

Effect of Hesperidin on serum Heart Marker, Myocardial Tissues Parameter and Histopathological of Heart in isoproterenol induced Myocardial infarction in Diabetic Rats

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Abstract

Present study was designed to evaluate Hesperidin on serum heart marker, myocardial tissues parameter and histopathology of heart in isoproterenol induced myocardial infarction in normal and Streptozotocin-Nicotinamide induced diabetic in rats. Hesperidin (100 mg/kg, p.o) was administered for 28 days in rats injected with single dose of Streptozotocin (65 mg/kg, i.p, STZ) and Nicotinamide (110 mg/kg, i.p, NIC) and after Isoproterenol (200mg/kg, s.c., ISO) induced myocardial infarction in rats on 29th and 30th day. At the end of experimental period (i.e. on the day 31) blood samples were collected and animals were euthanized. A heart tissue sample of each rat was collected and glycogen and nitrite carried out for further estimations. Administration of STZ–NIC in rats showed a significant ($P<0.001$) increased in the levels of serum glucose, glycosylated heamoglobin (HbA1c), creatine kinase (CK), Glutamate oxaloacetate transferase (GOT), glycogen and nitrite whereas the levels of myocardial infarct size was found low to be significant ($p<0.05$). Treatment with Hesperidin (HES) significantly decreased change HbA1c, glucose level and no change in glycogen but significantly reduced CK ($p<0.05$), GOT ($P<0.01$) and nitrite ($p<0.01$) in compared to diabetic control group. The myocardial fiber, heavy neutrophil infiltration and cellular edema than non diabetic rats. The HES treated infarction in diabetic rats also led to severe splaying of muscle diabetic rats exhibited reduction in necrosis with less fragmentation of fibres as compared to diabetic control groups, which reflects the cardio protective effect of HES. This study concluded that HES at 100 mg/kg may show reduce experimentally induced myocardial infarction in type 2 diabetic rats.

***Keywords*:** Cardioprotective, Isoproterenol, Streptozotocin, Nicotinamide

Introduction

Three major metabolic abnormalities contribute to the development of hyperglycemia in Type 2 diabetes mellitus such as impaired insulin secretion in response to glucose, increased hepatic glucose production and decreased insulin-stimulated glucose uptake in peripheral tissues. The latter 2 abnormalities are primarily due to insulin resistance (Kahn and Porte,1990; Leibowitz,1990). Cardiovascular disease is one of the leading causes of death in the western world and diabetes mellitus has been identified as a primary risk factor (Uemura *et a*l.,2001) due to which there is alteration in vascular responsiveness to several vasoconstrictors and vasodilators (Senses *et al*.,2001). Recently, a protective effect of Hesperidin against oxidative stress in liver and kidney of diabetic rabbits (Gumieniczek,2003) has been reported.

Hesperidin (HES) is an abundant and inexpensive byproduct of Citrus cultivation and isolated from the ordinary orange Citrus aurantium and other species of the genus Citrus (family: Rutaceae). It is reported to have anti-allergic, radio protective, immunomodulator, anti-hypertensive and anti-oxidant properties. When hesperidin is administered orally, it is hydrolyzed by intestinal micro flora to yield a major active metabolite hesperidin. So far the effect of Hesperidin on experimentally induced myocardial infarction in type 2 diabetic rats has not been studied. Hence, the purpose of the present study was to instigate the effect of Hesperidin treatment on serum heart marker, heart tissue parameter and histopathological alteration in Isoproterenol Induced myocardial infarction in type 2 diabetic rats.

Materials and Methods

Drugs and Chemicals

Hesperidin was obtained from ACROS Lab, US. STZ and NIC were obtained from SIGMA, St. Louis, MO, USA. All other chemicals and reagents used in the study were of analytical grade.

Experimental Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Dharmaj Degree Pharmacy College, Anand. Sprague Dawley rats (210 ± 15 g) were housed in-group of 3 animals per cage and maintained under standardized laboratory conditions (12- h light/dark cycle, 24°C) and provided free access to palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt., Pune) and purified drinking water *ad libitium*.

Experimental Induction of Type 2 Diabetes in Rats

Type 2 Diabetes was induced in rats by a single intraperitoneal (i.p) injection of Streptozotocin (65 mg/kg, STZ) in overnight fasting rats or mice followed by the i.p administration of Nicotinamide (110 mg/kg, NIC) after 15 minutes. STZ was dissolved in citrate buffer (pH 4.5) and NIC was dissolved in normal saline. After 7 days following STZ and NIC administration, blood was collected from retro-orbital puncture and serum samples were analyzed for blood glucose (Masiello *et al*.,1998). Animals showing fasting blood glucose higher than 300 mg/dL were considered as diabetic and used for the further study. Hesperidin (100 mg/kg, p.o) was administered for 28 days in diabetic rats and after isoproterenol induced myocardial infarction in rats on 29th and 30th day.

At the end of experimental period (i.e. on the day 31) blood samples were collected and animals were euthanized. A heart tissue sample of each rat was collected and carried out for further estimations.

Experimental Protocol

Animals were divided into following groups, each group containing 6 animals and the treatment period for whole study was 4 weeks.

Group 1: Non-diabetic control [0.5 % Sodium CMC (1 ml/kg/day, p.o) as vehicle for 4 weeks and (ND-CON)] and normal saline subcutaneously on 29th and 30th day.

Group 2: STZ-NIC diabetic control [0.5 % Sodium CMC (1 ml/kg/day, p.o) as vehicle for 4 weeks (D-CON)] and received ISO (200mg/kg, s.c.) on 29th and 30th day in normal saline.

Group 3: Non-diabetic control treated with HES (100 mg/kg/day, p.o) as a suspension [0.5 % Sodium CMC for 4 weeks (ND-HES)] and normal saline subcutaneously on 29th and 30th day.

Group 4: STZ-NIC diabetic rats treated with HES (100 mg/kg/day, p.o) as a suspension [0.5 % Sodium CMC for 4 weeks (D-HES)] and received ISO (200mg/kg, s.c.) on 29th and 30th day in normal saline.

Biochemical Estimation

Characterization of Type 2 Diabetes Model

Type 2 diabetes was confirmed by measuring fasting serum glucose using standard diagnostic kit (SPAN diagnostics Pvt., India) and the degree of uncontrolled diabetic state was confirmed by measuring HbA1c (Ion Exchange Resin method). After 4 weeks, diabetes was confirmed by measuring glucose and HbA1c as mentioned above.

Estimation of Serum Markers

On 4th week blood samples were collected from retro-orbital plexus under light ether anesthesia and centrifuged at 2500 rpm for 20 minutes to separate serum. Glucose, HbA1c, CK and GOT were estimated using diagnostic kits (SPAN Diagnostics Pvt. India). *In vitro* quantitative determination of the activity of myocardial glycogen and myocardial nitrite (Guevara *et al*.,1998) levels.

Histological Examination

After decapitation, the heart was rapidly dissected out and washed immediately with saline and fixed in 8% buffered formalin. Hearts which were stored in 8% formalin were embedded in paraffin, sections cut at 5 µm and were stained with haematoxyline and eosin. The sections of the heart were observed under microscope (Olympus BX8) for histological changes.

Statistical Analysis

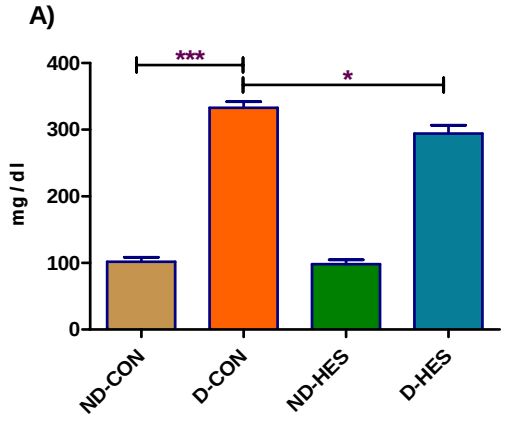
All of the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using a computer-based

fitting program (Prism, Graphpad 5). Differences were considered to be statistically significant when $p < 0.05$.

Results and Discussion

Characterization of Type 2 Diabetes

Single intraperitoneal (i.p) injection of Streptozotocin (65mg/kg) followed by i.p administration of Nicotinamide (110 mg/kg) to rats produced severe hyperglycemia and increased HbA1c in 70 to 80 % the animals (Figure 1). The levels of glucose and HbA1c was significant (P<0.05) decreased after treatment with HES (100 mg/kg, p.o) alone and combination with HES (100 mg/kg, p.o) as compared to DB-CON rats.



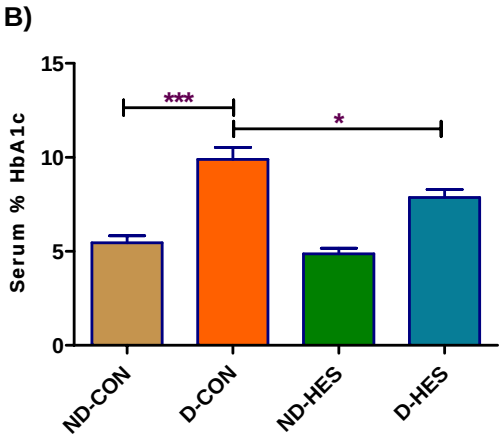


Fig.1: Effect of Hesperidin (100 mg/kg/day, p.o) on changes in serum glucose and HbA1c level in normal and STZ-NIC induced diabetic rats.

Body Weight and Heart Weight

Final body Weight of control animals was significant (P < 0.05) increased as compared to initial body weight. There was a significant reduction in final body weight as compared to initial body weight of D-CON diabetic group (Table 1). Hesperidin treatment had no significant effect on the body weight of D-CON group animals. There was a significant (P < 0.05) increased in heart weight of diabetic rats (D-CON). HES treatment could prevent increase in heart weight in diabetic rats (D-CON). Heart to body weight ratio of the entire group is show in (Table -1).

Table -1: Effect of Hesperidin (100 mg/kg/day, p.o) on changes in body weight, heart weight and heart to body weight ratio after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.

Groups	Body Weight						Heart Weight (gm)			Heart to Body Weight ratio		
	Initial			Final								
ND-CON	240.6	±	12.5	261.6	±	15.4#	0.872	±	0.021	0.00333	±	0.00064
D-CON	249.2	±	17.4	224.4	±	16.1#	0.973	±	0.019*	0.00433	±	0.00027*
ND-HES	236.9	±	19.9	248.9	±	15.6	0.862	±	0.038	0.00346	±	0.00039
D-HES	242.6	±	18.4	254.3	±	14.8	0.942	±	0.046*	0.00370	±	0.00049

Values are expressed as mean ± SEM for six animals in the group. * P<0.05 compared to respective control group and # P<0.05 compared to initial weight.

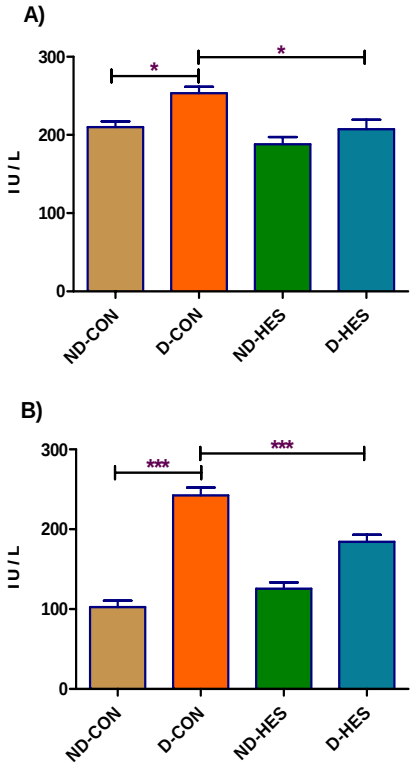


Fig. 2. Effect of Hesperidin (100 mg/kg/day, p.o) on changes in serum Creatine kinase (CK) and Glutamate oxalatoacetate transferase (GOT) level after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.

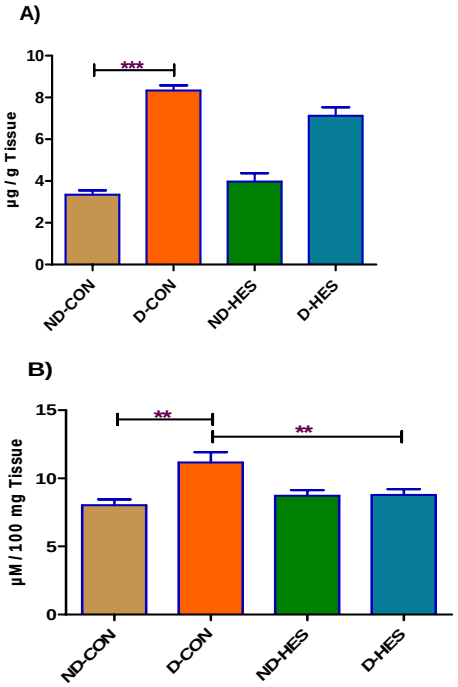


Fig.3. Effect of Hesperidin (100 mg/kg/day, p.o) on myocardial changes in Glycogen (A) and Nitrite (B) level after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.

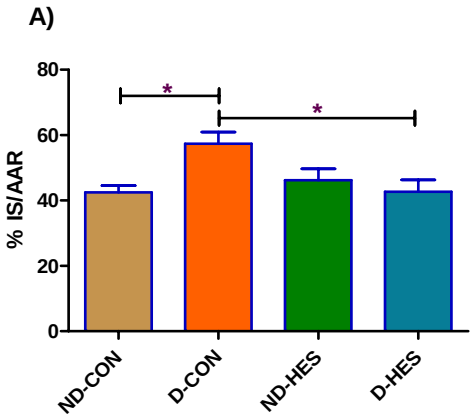


Fig.4. Effect of Hesperidin (100mg/kg/day, p.o) on myocardial infact size changes after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.

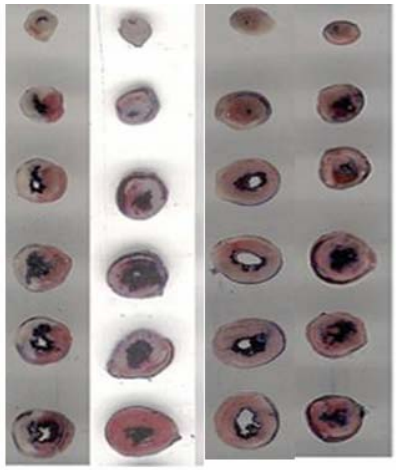


Fig.5. Effect of Hesperidin (100 mg/kg/day, p.o) on TTC stained myocardial sections changes after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.

Histopathology of Heart

The photomicrographs revealed that induction of myocardial infarction caused more necrotic damage along with focal loss and fragmentation of muscle fibres of myocardial in diabetic rats (D-CON) than non diabetic rats (ND-CON) (fig. 6). The myocardial infarction in diabetic rats (D-CON) also led to severe splaying of muscle fiber, heavy neutrophil

infiltration and cellular edema than non diabetic rats (ND-CON). The HES treated diabetic rats (D-HES) exhibited reduction in necrosis with less fragmentation of fibres as compared to D-CON groups, which reflects the cardio protective effect of HES (Fig. 6). However, HES treatment could protect myocardial infarction against in non diabetic rats (ND-HES).

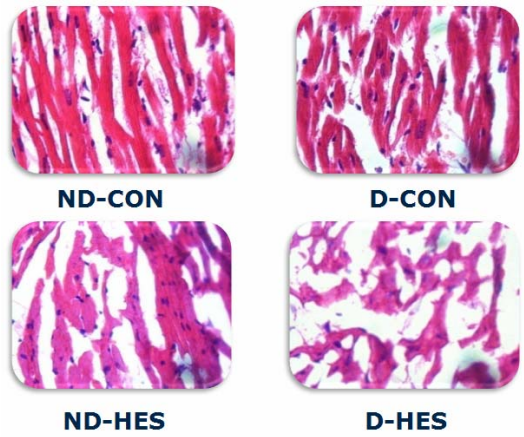


Fig. 6: Effect of Hesperidin (100 mg/kg/day, p.o) on light micrographs of histopathological section of heart changes after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.

Discussion

The present study was under taken with the objective of exploring the Hesperidin Reduces on experimently induced myocardial infarction in diabetic rats. Recent studies have suggested that prevalence of type 2 diabetes is rapidly increasing. Heart failure of myocardial infarction or ischemic origin is more frequent and severe in patients with diabetes. Diabetes is an independent risk factor for cardiac failure (Kannel and Mcgee,1979), although its detrimental impact on the myocardium remains to be identified. The significant amount of myocytes loss in this model of non insulin dependent diabetes mellitus is consistent with a greater vulnerability of the diabetic heart to cardiac processes.

The release of ROS in the early phase of myocardial, in combination with the infarction induced decrease in anti-oxidant activity, renders the myocardium vulnerable. Previous studies proved that, ROS produced during myocardial infarction could trigger myocyte apoptosis by activating MAPK and produces DNA damage by activation of the nuclear enzyme poly (ADP ribose) polymerase, which consumes cellular Nicotinamide dinucleotide and adenosine triphosphate.

In the present study, an increase in the levels of serum glucose and HbA1c in STZ-NIC treated rats confirmed the induction of diabetes mellitus. Significant decreased was observed in the glucose and HbA1c level in diabetic rats after treatment with HES (100 mg/kg) when compared with D-CON rats at the end of experimental period. There was a significant increase in heart weight in STZ-NIC diabetic rats which may be due to cardiomyopathy associated with diabetes. It was reflected by increase in serum CK and GOT levels along with heart weight to body weight ratio. Hesperidin could protect the heart from cardiomyopathy associated with STZ-NIC diabetes. This may be the reason for decreased serum CK and GOT level in D-HES group. Myocardial infarction causes further reduction in nitric oxide due to endothelial dysfunction. Hesperidin reduced myocardial infarct size in STZ-NIC diabetic rats. The glycogen deposition in heart is increased in STZ-NIC diabetic rats which may be due to reduction in glucose utilization. HES reduced cardiac glycogen content in STZ-NIC diabetic rats (D-HES) by increasing glucose utilization after myocardial infarction. Therefore, another possibility for cardioprotection by HES may be shifting of energy substrate metabolism from fatty acid to glucose. Reduction in oxidative stress and increase in NO level in Hesperidin treated control group showed significant improvement in CK and GOT.

There may be several mechanisms for cardioprotective by HES against myocardial infarction. It may be due to improvement in NO availability in STZ-NIC diabetic rats. Administration of STZ caused increase in serum CK, GOT and Hesperidin (100 mg/kg, p.o) could reduce them. This study concluded that HES at 100 mg/kg may show reduced on experimentally induced myocardial infarction in diabetic rats.

References

Kahn, S.E. and Porte, D.J.1990. The pathophysiology of type II (noninsulin-dependent) diabetes *mellitus*: Implications for treatment. In: Rifkin, H., Porte, D.J., eds. Ellenberg and Rijkin's Diabetes Mellitus:

Theory and Practice. New York: Elsevier Science. 436-456.

Leibowitz, H.E. 1990. Oral hypoglycemic agents. In: Rifkin H, Porte, D.J., eds. Ellenberg nd Rijkin's Diabetes Mellitus: Theory and Practice. New York: Elsevier Science. 554-574.

Uemura, S., Matsushita, H., Li. W., Glassford, A.J., Asagami, T., Lee, KH., *et al*.2001. *Diabetes mellitus* enhances vascular matrix metalloproteinase activity: role of oxidative stress. *Circ Res*., 88:1291– 1298.

Senses, V., Ozyazgan, S., Ince, E., Tuncdemir, M, Kaya, F., Ozturk, M., *et al*.,2001. Effect of 5-aminoimidazole-4-carboxamide riboside (AICA-r) on isolated thoracic aorta responses in streptozotocin-diabetic rats. *J. Basic. Clin Physiol Pharmacol*., 12:227– 248.

Gumieniczek, A.2003. Effect of the new thiazolidinedione - Hesperidin on the development of oxidative stress in liver and kidney of diabetic rabbits. *Life Sci*., 74:553-562.

Masiello, P., Broca, C., Gross, R., Roye, M., Manteghetti, M., Hillaire-Buys, D., Novelli, M. and Ribes, G. 1998. Experimental NIDDM: development of a new model in adult rats administered Streptozotocin and Nicotinamide. *Diabetes*, 47, 224–229.

Guevara, S. M., Iwanejko, J., Dembinaka-Kiec, A., Pankiewicz, J., Wanat A, Anna P, *et al*., 1998. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. *Clin Chim Acta*., 274: 177-88.

Kannel, W. B., Mcgee, D. L., 1979. Diabetes and cardiovascular disease. The Framingham study. *JAMA*, 241: 2035-2038.

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Original Article

Azotobacter population in Rhizosphere and Non-Rhizosphere sediments of Tondi Coast

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Abstract

Tondi marine region is shallow and contains more vegetation. The samples of rhizosphere and non-rhizosphere sediments were collected from Tondi marine region. The presence of *Azotobacter* population was observed in all the samples collected from both rhizosphere and non-rhizosphere sediments. In non-rhizosphere sediments, the population of *Azotobacter* ranged between 3.1 X 10^2 and 6.9 X 10^3 CFU g^{-1}, whereas in rhizosphere sediments, *Azotobacter* population was varying from 3.2 X 10^2 to 10.7 X 10^3 CFU g^{-1}. This study revealed that the maximum *Azotobacter* population was recorded rhizosphere sediment of Tondi marine region during monsoon season.

Key words: Tondi marine; *Azotobacter*; rhizosphere; non-rhizosphere.

Introduction

Tondi marine region is shallow and contains more vegetation. The samples of rhizosphere and non-rhizosphere sediments were collected from Tondi marine region. The presence of *Azotobacter* population was observed in all the samples collected from both rhizosphere and non-rhizosphere sediments. In non-rhizosphere sediments, the population of *Azotobacter* ranged between 3.1 X 10^2 and 6.9 X 10^3 CFU g^{-1}, whereas in rhizosphere sediments, *Azotobacter* population was varying from 3.2 X 10^2 to 10.7 X 10^3 CFU g^{-1}. This study revealed that the maximum *Azotobacter* population was recorded rhizosphere sediment of Tondi marine region during monsoon season.

Materials and Methods

Collection and processing of Sample:

The sediment samples were collected from rhizosphere and non-rhizosphere region every month for a period of one year from January 2004 to December 2004. Sediment samples were collected in sterile polyethylene bags employing precleaned Peterson grab from non seagrass region (sediment) and from the seagrass region by coring (rhizosphere sediment). Collected samples were placed properly in the ice boxes and transported safely to the laboratory for the further studies (Swarnakumar *et a*., 2007).

Isolation and Identification of Azotobacter sp:

According to Zehr *et al*., (1998) methods followed by the sediments were serially diluted and plated on Burk's agar medium (HiMedia) by spread plate technique. The colonies were recorded after the incubation in an inverted position for 48 hrs at 30°C. It was identified and confirmed by the method of Bergey's Manual of Systematic Bacteriology (Williams *et al*.,1994). The culture characteristic of *Azotobacter* sp was also noted on the Burk's media for its distribution.

Screening: (Shehata and El-Khawas, 2003)

The isolated *Azotobacter* sp were subjected to different physical parameters like sugar concentration, pH, and temperature influence on the growth and also observed morphological differences (Cappuccino and Sherman. 1996). Hence the strains were grown in varying sugar (Sucrose) concentrations like 0.5, 1.0, 2.0, 3.0, 4.0 % and the influence of sugar was recorded with Burk's broth using spectrophotometer at 520 nm. Similarly the pH (5.0, 6.0, 7.0, 8.0 and 9.0) and temperature (20, 28, 37 and 45°C) influence were recorded.

Results and Discussion

Biological nitrogen fixation has gained recognition as an important source of nitrogen for supporting oceanic primary production and other organisms (Zehr *et al*., 1998). It is recognized that microbial communities play a crucial roles in the nutrient cycling necessary to maintain ecosystem health (Ellis *et al*.,2003). The research of Ellis *et al*. (2003) and Grandlic *et al*. (2006) indicated the abundance, diversity,

and function of microbial communities in the different environment. The ability of *Azotobacter* to fix nitrogen was established by Beijerinck in 1901. However, it has been shown that they fix nitrogen gas only in the absence nitrogenous compounds Lakshmana perumalsamy,1987; Grandlic *et al*.,2006). *Azotobacter* population in non-rhizosphere sediments of Tondi were ranged between 3.1 X 10^2 and 6.9 X 10^3 CFU g^{-1} during 2004. It was observed as minimum (3.1 X 10^2 CFU/g^{-1}) during summer (June 2004) and the maximum (6.9 X 10^3 CFU g^{-1}) was recorded during monsoon (November 2004).

In rhizosphere sediments it was varying from 3.2 X 10^2 to 10.7 X 10^3 CFU/g^{-1}, in which minimum *Azotobacter* load (3.2 X 10^2 CFU g^{-1}) was recorded during pre monsoon (August 2004) but maximum (10.7 X 10^3 CFU/g^{-1}) was observed during monsoon season (Nov. 2004) (Fig. 1).

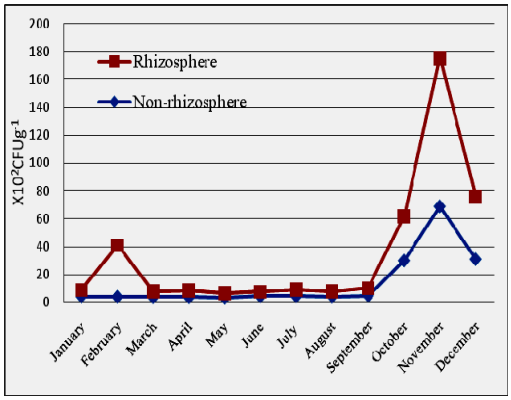

Fig-1: *Azotobacter* population in sediments of Tondi marine region during 2004

From the reports, investigations on the occurrence, distribution and seasonal variations of the *Azotobacter* population in the coastal water are very meager and scattered that is 10^6/g was reported in the Pichavaram mangrove (Purushothaman,1999; Swarnakumar *et al*., 2007). Kawai and Sugahara (1971) observed the population ratio between 10^2/g in Surugu Bay and 10^4/g in Sagami Bay culture ponds.

Hofer's (1948) study and Bergey's manual reported about the differences in the culture character and colour of the colony of *Azotobacter* sp. The culture characteristics of *Azotobacter* isolates were recorded in the Burk's media and found that there were six types of colony morphology and four different colour colonies. Most of the isolates were observed with smooth and watery type colony morphology (63.3 %), and watery, white colour colonies (66.7 %) (Table -1).

Table -1: Characteristics and Colour of the *Azotobacter* isolates on selective media

Culture character of the colonies	Isolates %	Colour of the colony	Isolates in %
Smooth, watery/dew drops	63.3	Watery/white	66.7
Smooth, circular	13.3	Pale white	16.7
Rhizoid	6.7	Pink/reddish	6.7
Large circular, flat	3.3	Pale yellow	10.0
Small,circular	6.7		
Pin head	6.7		

The growth influence of *Azotobacter* isolates were observed using parameters like sugar, pH and temperature (Fig.2). It was revealed that, there was a significant growth at 1 (76.6%), 2 (86.6%) and 3 % (90.0%) of glucose concentrations. Likewise, a remarkable growth was recorded at pH 8.0 (90.0 %) and temperature 28 °C (86.6 %).

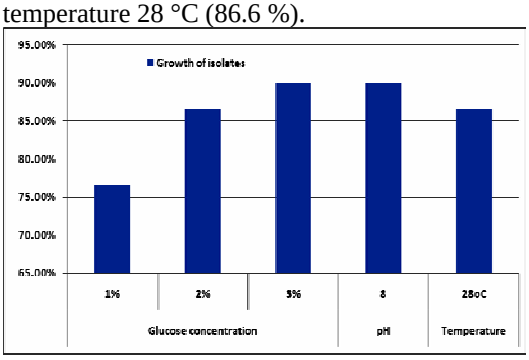

Fig- 2: The growth influence of *Azotobacter* by various parameters

Saline environments harbor taxonomically diverse bacterial groups, which exhibit modified physiological and structural characteristics under prevailing saline conditions (Zahran. 1997). The majority of these bacteria can osmoregulate by synthesizing specific compatible organic osmolytes such as glutamine, proline and glycine betaine and a few of them accumulate inorganic solutes such as Na^+, K^+ and Mg^{2+}. Evidence has accumulated that the bacteria including *Azotobacter* are essential elements in saline environment because of their activity such as degradation of plant remains, nitrogen fixation and production of active metabolites (Page *et al*,. 1991).

Conclusion

Azotobacter population was more and their distribution was good in both rhizosphere and non-rhizosphere sediments of Tondi marine region. This is may be due to the accumulation of the seagrass fragments and seaweeds in sediments, decomposition of the seagrass animals, transfer of nutrients from near the shore and anthropogenic activities and terrestrial run off from the agricultural and domestic wastes.

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References

Zehr, J.P., Mellon, M.T and Zani, S.1998. New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. *Appl.Environ. Microbiol*., 64: 3444-3450.

Shehata, M.M and El-Khawas, S.A.2003. Effect of two biofertilizers on growth parameters, yield characters, nitrogenous components, nucleic acids content, minerals, oil content, protein profiles and DNA banding pattern of sunflower (*Helianthus annuus* L. cv. *vedock*) yield. *Pakistan J. Biol. Sci*., 6 (14): 1257-1268.

Ravikumar, S. 1995. Nitrogen - Fixing *Azotobacter* from the mangrove habitat and their use as biofertilizer. Ph. D. thesis, Annamalai University, Annamalai Nagar,pp.113.

Bergey's, 1994. Manual of Systematic Bacteriology, 9th *Eds*. (Williams, S.T., Sharpe, M.E & Holt, J.G.) Williams and Wilkins, Baltimore, USA.

Cappuccino, J.G and Sherman, N. 2006. Microbiology - A laboratory manual. 6th edition, Pearson education, USA, pp.150-175.

Pace, N.R.1997. A molecular view of microbial diversity and the biosphere. *Science*, 276: 734-740.

Ellis, R.J., Morgan, P., Weightman, A.J and Fry, J.C.2003. Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil, *Appl. Environ. Microbiol*., 69: 3223-3230.

Grandlic, C.J, Geib, I., Pilon, R and Sandrin, T.R.2006. Lead pollution in a large, prairie-pothole lake (Rush Lake, WI, USA): Effects on abundance and community structure of indigenous sediment bacteria. *Environ. Pollution*, 144: 119-126.

Lakshmanaperumalsamy, P.1987. Nitrogen fixing bacteria, *Azotobacter* sp. In aquatic sediment, *Fish Technol*., 24 (2): 126 - 128.

Purushothaman, A.1999. Studies on bacterial ensemble in a developed *vis-a-vis* developing mangrove ecosystem. Ph. D thesis, Centre of Advanced Study in Marine biology, Parangipettai, pp.9-50.

Swarnakumar, N.S., Thangaradjou, T., Sivakumar, K and Kannan, L. 2007. Distribution of total heterotrophic bacteria in the coastal waters of the Great Nicobar with special reference to human pathogens, *Natl. Acad. Sci. Lett*., 30: 129-137.

Hofer, A.W.1948. *Azotobacteriaceae*. In Bergey's manual of determinative bacteriology, 6th ed. (Breed RS, Murray EGD & Hitchens AP) The Williams and Wilkins Co, Baltimore, Md. Pp. 219-222.

Zahran, H.H.1997. Diversity, adaptation and activity of the bacterial flora in saline environments, *Biology and Fertility of Soils*., 25(3): 211-223.

Beijerinck, M.W.1901. Ueber Oligonitrophile Mikroben, Centr. F. Bakt., 7: 561-582.

Page, W.J and Shivprasad, S.1991. *Azotobacter salinestris* sp. nov., a sodium-dependent, microaerophili, and aeroadaptive nitrogen-fixing bacterium, *Int. J. Syst. Bacteriol*., 41: 369-376.

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Original Article

Application of statistical methods to optimize medium for increased yield of Oyster Mushroom (*Pleurotus ostreatus*)

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Abstract

The aim of this study is investigating sustainable alternatives to grow Oyster mushroom (*Pleurotus ostreatus*) using paddy straw, bagasse, wheat bran, urea and humic acid at varying composition. The study optimizes the composition of the substrates, other than the paddy which is the main raw material, for the maximum yield of *Pleurotus ostreatus* and analyses the nutrient content of the biomass produced. Bagasse composition (17.5g/l - 32.5g/l), Wheat bran composition (3.5g/l – 6.5 g/l), Urea composition (3.0 g/l -7.0 g/l) and Humic acid (2% - 6%) were chosen as the process variables for optimization. A 5 level 4 variable central composite design was used to evaluate the effects of these parameters on the Yield of *Pleurotus ostreatus*. After the process of optimization, the significant interaction among the process variables studied. Humic acid is the key role in the determination of the yield. Depending on the different process parameters the yield of mushroom varied from 74 – 204g. Optimum process parameters for maximum yield of *Pleurotus ostreatus* were found to be Bagasse 21.25g/l, Wheat bran 3.5g/l, Urea 5.0g/l and Humic acid 4%. The process parameters also shows significant effect on yield, productivity and biological efficiency. Mycelial colonization of compost bags and subsequent growth of oyster mushroom was faster in high Humic acid-based substrates. Hence they produced larger and firmer fruiting bodies. The response surface methodology provided here can be used as a strategy to grow Oyster mushrooms under adverse conditions and limited resources.

Key words: Oyster mushroom*; Pleurotus ostreatus;* Humic acid; Wheat bran

Introduction

The Oyster mushroom, or *Pleurotus ostreatus*, is a common edible mushroom, long cultivated in Asia, it is now cultivated around the world for food. They are commonly grown in mushroom houses but require a more humidity and fresh air than other variety. They grow well on a range of agricultural and wood waste products including hardwood chips, chopped cereal straws or corn cobs. The usage of substrate as fertilizer after mushroom production is more commonly seen if straw is used as a key ingredient in growth medium. Oyster mushrooms can also be used industrially for mycoremediation purposes. Fasidi and kadiri (1993) reported the growth of mushroom to be affected by moisture content, temperature, pH and light intensity when it was grown on lignocelluloses wastes. Ragini Bisaria *et al.* (1983) suggested the usage of Lignocelluloses for microbial conversions. The intra and extracellular contents of vitamins in *Pleurotus ostreatus* were studied in the course of submerged cultivation by Solomko and Eliseeva,(1988). The dry mycelium of *P. ostreatus* obtained after the cultivation revealed the production of thiamin (vitamin B_1), riboflavin (vitamin B_2), niacin (vitamin B_5), pyridoxine (vitamin B_6) and biotin (vitamin B_7). *Pleurotus* sp are medicinal mushrooms, exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial, hypocholesterolic and immunomodulation activities (Cohen *et al*., 2002).

Humic acid is a condensed, refractive mixture of aromatic organic acids which contains sulfur, nitrogen, phosphorus and metals such as Cu, Mg, Cu, Zn. Humus is reported to be a chemical complex, which is resistant mixture of brown or dark brown amorphous, hydrophilic, acidic, partly aromatic organic substances modified from the original tissues or synthesized by the various soil organisms that range in molecular weight from a few hundred to several thousand (Kononova *et al*., 1966). Identification of complex structure of soil humus consisting of humic materials such as humic acid, fulvic acid and humin aided its application in many field (Schnitzer,1978). *Pleurotus ostreatus* was cultured on a synthetic medium with growth regulators (Vinklarkova and Sladky,1978).

Humic acid was found to be insoluble at greater pH values. Hence the extraction and separation of various humic substances are carried out using 0.1-0.5N NaOH (Stevenson and Schnitzer, 1982). The negatively charged carboxylic and phenolic groups in the colloidal surfaces of humus are pH dependent (Schnitzer, 1986). The efficiency of humic acid in improving the N and P contents thereby aiding the increase in crop yield was revealed by Brannon and Somers, (1985).

The highest yield of fruiting bodies was obtained using a mixture of date waste and rice straw as substrates (Jwanny *et al*.,1995).The protein quality of edible mushrooms and Amino acid evaluation of *Pleurotus* sp. cultivated in banana leaves and a mixture of banana leaf and bagasse was studied by Ranzani and Sturion, (1998). Saw dust as substrate produced highest yield, biological efficiency and number of fruiting bodies for oyster mushroom cultivation as shown by Shah *et al*.(2004). Utilization of whey permeate and application of Response surface analysis (RSA) was investigated to determine the combination of substrate concentration, temperature and pH producing maximal mycelial extension rate under solid state cultivation (Bhak *et al*., 2005). Determination of nutritive value and yield performance of three types of oyster mushroom *P.eryngii , P.ostreatus, P.Sajor-caju* cultivated on wheat stalk revealed the production of ligninolytic enzymes which finds significant importance in the biodegradation of organopollutants, xenobiotics and industrial contaminants (Dundar *et al*., 2008). Although mushroom culture is one of the oldest microbial foods of man and the first solid-state fermentation product, the basic research of microbial technology has not been applied to significant extent. The present study investigates the production of Oyster mushroom by providing various combination of substrates and optimizing the composition of the substrate for maximum yield by Response Surface Methodology.

Materials and Methods

Inoculum preparation

The main culture of *Pleurotus ostreatus* was obtained from Thirukalukundram mushroom farm.

For the propagation of the main culture, 2.0% Malt-Extract Agar (MEA) was used. MEA plates were inoculated with a mycelium/agar plug (5-mm-diameter) of a young, actively growing margin of the colony. Prior to its use as an inoculum for grain spawn, a mycelium/agar plug was inoculated at the center of the plate and incubated at 25°C in the dark for seven days.

Spawn preparation

200g sorghum was used as substrate for spawn production. The grain was soaked in 500 ml water. Excess water was drained and grains were shredded into tiny pieces. 50 g grain was placed in polythene bags and held in place by rubber bands, it is sterilized 121ºC for 20 min. After cooling, each bag was inoculated with spawns and incubated at 25ºC in full darkness for two weeks to enable the mycelia to permeate.

Conditions of cultivation

Paddy straw was used as a main material in this study for cultivation of oyster mushrooms. Paddy straw was soaked in water filled plastic buckets for 16 h. After which, it is crushed and dried. The crushed straw is treated with different composition of bagasse, wheat bran, urea and humic acid. pH of the substrate mixtures were maintained at 6.0. Compost medium was mixed manually. The mixture of varying compositions were packed in polythene bags and sterilized. After sterilization, the substrate is semi dried, inoculated with the spawns in alternate layers and incubated at 25°C in dark for 2 weeks for the ramnification of mushroom mycelia. The culture rooms were damped by spraying the top of compost with water once a day. This maintains the relative humidity of 80 %. After the development of mycelium on compost bags, they were torn and maintained at 28 °C with adequate aeration, watering and high humidity to allow the fruiting bodies to emerge. The harvesting was done in 3 flushes of 1 week intervals. After the 2nd flush, the substrate was turned upside down and regularly watered to harvest the 3rd flush.

Moisture content

The fresh weight of each mushroom sample was determined by chemical balance. The sample with 5 g initial weight was oven dried separately at 95°C for 24 h till constant weight is attained. The loss in weight obtained after drying was regarded as the moisture content in percentage (Manzi *et al*., 1999), which is calculated as follows using

$$Moisture\ \% = \frac{(W_0 - W_1)}{W_0} \times 100 \quad (1)$$

Lipid

3.0g of dried mushroom sample was extracted with 25 cm^3 of petroleum ether in a soxhlet extractor for 16 h. The extract was evaporated to dryness in a weighed flask using a vacuum evaporator. The weighed flask was dried in the oven at 105°C for 30 min. The weight of the extract was recorded after cooling in desiccator. The difference between the initial and final weights was regarded as the lipid content of the sample (Parent and Thoen,1977). Crude fat was calculated by

$$Crude\ fat\ (\%) = \frac{W_{fat,S} \times 100}{Ws} \quad (2)$$

Crude fibre

3g dried and fat free mushroom sample was taken in a 1000 ml beaker and 200 ml of 1.25% H□SO□ were added. The level of beaker was marked. The contents of the beaker were boiled for 30 min with constant stirring; also the level of the water was supplemented. Contents were given 3 washings with hot water (150 ml) until it was acid free. The procedure was repeated with 1.25% NaOH. The alkali free residue was carefully transferred to a crucible and dried in an oven at 100°C for 4 hours until constant weight was obtained. The contents were heated on oxidizing (blue) flame until smoke ceased to come out of sample. The sample was placed in a muffle furnace at 550°c for 4 hours until a grey ash was obtained, cooled in desiccators and weighed (AOAC, 1990). The difference in weight gives crude fibre as calculated by

$$Crude\ fibre\ (\%) = \frac{\Delta W_{\text{after ignition}}\ (g)}{W_{\text{sample}}\ (g)} \times 100 \quad (3)$$

Ash content

3.0 g of dried mushroom sample were taken in a crucible and headed on oxidizing flame till smoke subsided. The crucible was transferred to muffle furnace at 550°C for 6 hours. The sample was cooled in desiccators and weighed (Manzi *et al*., 2001). The ash content in the sample was calculated by

$$Ash\ (\%) = \frac{W_{\text{Ash,sample}}\ (g)}{W_{\text{sample}}\ (g)} \times 100$$

Protein content

0.5 g of the powdered and mushroom sample was extracted with 50.0 cm^3 of 2.5% NaCl in a water-bath at 60°C for 1 h. The extract was filtered out and treated with 3% copper acetate to precipitate the protein. The precipitated protein was centrifuged and dissolved in 0.1 M NaOH. The quantity of protein in the alkaline solution was determined using the folins-phenol method (Kadiri and Fasidi,1990).

Results and Discussion

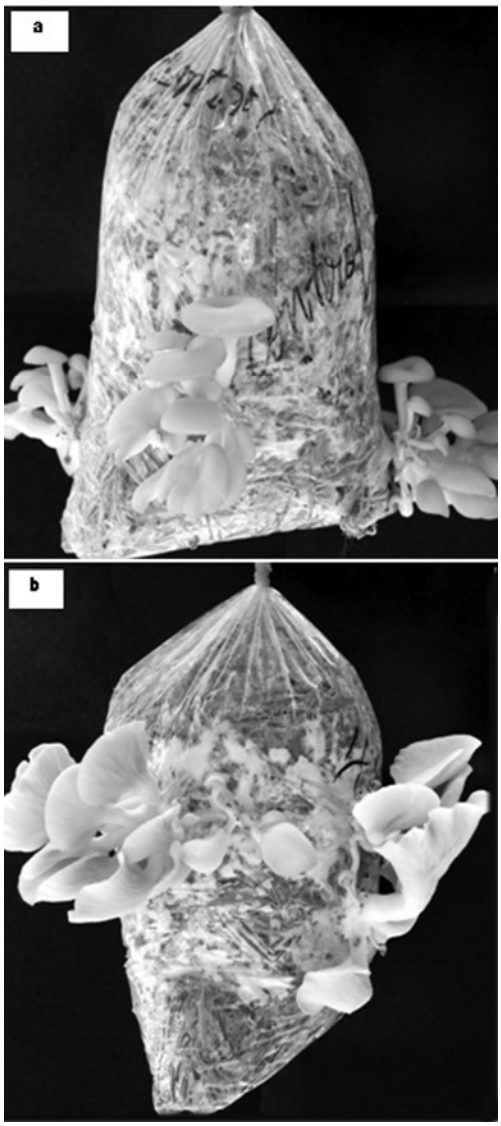
Mushroom growth

It was seen from the study spawn running, pinhead formation and fruiting body formation took place in about 2 -3 weeks after inoculation, 7-8 days after spawn running and 3-5 weeks after pin head formation. Similar findings were reported by (Tan *et al*., 1981; Ahmed *et al*., 1986; Quimio *et al*., 1976,1978). The mushroom growth in control and the substrate with 4 % humic acid is shown in Figure 1.a and Figure 1.b.

Experimental design

Adoption of Central Composite Design employed here requires prior knowledge on the upper and lower limits of the parameters, awareness of the cultivation process and its factors. The preliminary trails indicated that amount of Bagasse, Wheat bran, Urea and volume of Humic acid were significant variables for the cultivation process. Hence, these four variables were chosen to obtain the optimum levels. The effect of composition trials on the yield of *Pleurotus ostreatus* is given in Figure 2. A four-factor, three-level Central Composite design was used to determine the optimal values for the factors. The central composite design (CCD) with a quadratic model was employed. Four independent variables namely Bagasse (17.5 g/l -30.0 g/l), Wheat bran composition (3 g/l–6 g/l), Urea composition (4 g/l-6 g/l) and Humic acid composition (4 – 6 ml) was chosen. Each independent variable had 4 levels which were –2, – 1, 0 and +1, +2. A total 30 different combinations (including eight star points and six replicates of centre point) were chose in random order according to a CCD configuration. The levels of variables and experimental design

matrix were presented in Table 1.a and Table 1.b.



The coded values of independent variables were found from equation

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad i = 1,2,\ldots,k \quad (5)$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point and ΔX is the step change. A second-order polynomial model was used to fit the quadratic, resulting in the equation,

$$Y_{pre} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_1^2 + \beta_6 x_2^2 + \beta_7 x_3^2 + \beta_8 x_4^2 + \beta_9 x_1 x_2 + \beta_{10} x_1 x_3 + \beta_{11} x_1 x_4 + \beta_{12} x_2 x_3 + \beta_{13} x_2 x_4 + \beta_{14} x_3 x_4$$

where Y_{pre} is the measured response, x_1, x_2, x_3, x_4 are the coded independent input variables, β_0 is the intercept term, $\beta_1, \beta_2, \beta_3, \beta_4$ are the linear coefficients showing the linear effects, $\beta_5, \beta_6, \beta_7, \beta_8$ are the quadratic coefficients showing the squared effects and $\beta_9, \beta_{10}, \beta_{11}, \beta_{12}, \beta_{13}, \beta_{14}$ are the cross product coefficients showing the interaction effects. Thirty experiments were performed in triplicate. The results obtained were submitted to analysis of variance on SAS package and the regression model was given as

$$Y_{pre} = 367.549 + 18.5x_1 - 286.778 x_2 + 64.292x_3 + 61.167 x_4 - 0.342 x_1^2 + 28.33 x_2^2 - 9.063x_3^2 + 2.188x_4^2 - 0.2667x_1x_2 + 0.8333x_1x_3 - 1.333x_1x_4 + 2.667x_2x_3 - 3.5x_2x_4 - 3.75x_3x_4 \quad (6)$$

where Y_{pre} is the Yield of *Pleurotus ostreatus* in this study and x_1, x_2, x_3, x_4 are the coded levels of independent input variables, amount of Bagasse, Wheat bran, Urea and volume of Humic acid respectively. The significance of each coefficient was determined by Student's t-test and P-value, which is listed in Table 2.a. The larger the magnitude of t-test and smaller the P-value, the more significant is the corresponding coefficient (Du, 2003). The regression model could be used to predict the future observations on the response Y corresponding to particular values of the regressor variables. During the prediction of new observations and estimation of the mean response at a given point, care must be taken about extrapolating beyond the region containing the original observations. The regression coefficient for the second order polynomial equations and results for the linear, quadratic and interaction term are discussed in detail. The optimum amount of Bagasse, Wheat bran, Urea and volume of Humic acid were obtained by solving the regression equation.

The results of the second-order response surface model in the form of analysis of variance (ANOVA) are given in Table 2.b. The Fisher's F-test ($F_{(14;15)} = S^2_m/S^2_s = 3804.614 > F_{t(14;15)} = 2.42$) with a very low probability value [($P_{model} > F$) < 5.37E-24] indicated the model was highly significant. The goodness of fit of the model was examined by determination coefficient (R^2 =0.9997), which implied that the sample variation of more than 99.97% was attributed to the variables and only 0.03 % of the total variance could not be explained by the model. A high value of correlation coefficient (R=99.99) signifies an excellent correlation between the independent variables. The adjusted determination coefficient (Adj R^2 = 0.9995) was also satisfactory to confirm the significance of the model. The residuals were examined to check the adequacy

of the model. The residuals were plotted against the predicted Y as shown in Figure 3. The ''horizontal band' indicated no abnormality, confirming the adequacy of the regression model (Draper *et al*., 1981). The amount of bagasse, urea and volume of humic acid added for the process of cultivation had a strong positive linear effect on the response. The amount of wheat bran and humic acid added showed a squared effect. However, humic acid addition was seen to be more significant compared to wheat bran. There was sufficient amount of interaction between bagasse- urea and wheat bran - urea, whereas the amount of wheat bran alone was less significant. The model predicted the maximum yield of 204.25 g, which appeared at the bagasse, Wheat bran, and Urea concentration of 21.25 g/l, 3.5 g/l, 5.0 g/l and Humic acid of 4 %, respectively. The subsequent experiments with the optimized conditions yielded consistent results with the prediction. The effects of four variables on the Yield of *Pleurotus ostreatus* were studied and the relevant conditions for the process were optimized.

Mushroom yield(Y), productivity (P %) and biological efficiency (BE %)

The maximum yield 204 g of Oyster mushroom was obtained on the nineteenth trial with 4 % humic acid. This finding is similar to Vaughan and Linehan, (1976) who reported enhancement of root growth and shoot growth in wheat plants by the application of humic acid. This was due to valuable byproducts released due to the microbial degradation of humic acid. The yield obtained was found to vary between the trials from 74 g to 204 g. Productivity was determined from the relation between mushroom fresh weight (M_F) and compost fresh weight (C_F).Biological efficiency was determined from the relation between mushroom fresh weight (M_F) and compost dry weight ($C_{D)}$ and is given by

$$P\% = \frac{M_F}{C_F} \times 100 \qquad (7)$$

$$BE\% = \frac{M_F}{C_D} \times 100 \qquad (8)$$

High productivity value of 18.47 % and biological efficiency of 27.16 % was observed for the nineteenth trial (Table 3). This was similar to the findings of Meire Cristina Nogueira de Andrade *et al*., (2007) who reported a productivity of 8.70 % and biological efficiency of 28.70 % in *Agaricus blazei* mushrooms grown in presence of *Trichoderma* sp.

Analysis of Nutrients

The protein content of the 19th trial was found to be 40 g, this was found to be more than the content of protein reported by Breene (1990) who illustrated the content of protein between 19 to 39 g in 100 g of dried matter. Fat value was 0.1 g in accordance with the value of 2.0 g in 100 g dry matter reported by Shah *et al*.(1997). 34.8 % dietary fibre was obtained from the cultivation of Pleurotus *ostreatus* by Justo *et al*. (1999), whereas our study reported a dietary content of 19.5 %.

Conclusion

The data obtained from the experiments demonstrated the strategies for enhancing production of *Pleurotus ostreatus.* The results of the model for mixture design experiments showed that the amount of bagasse, urea and percentage of humic acid added for the process of cultivation had a strong positive linear effect. The model predicted the maximum yield at bagasse, Wheat bran, and Urea concentration of 21.25 g/l, 3.5 g/l, 5.0 g/l and Humic acid of 4 % respectively. Bagasse- urea and wheat bran - urea composition showed sufficient amount of interaction, whereas wheat bran alone was less significant. However, humic acid addition was seen to be more significant compared to wheat bran and it had a positive influence on the yield of *Pleurotus ostreatus*. According to these results, the yield of 204.25 g predicted by the model agrees well with the experimental value of 204 g. This indicates the adequacy of the generated model in predicting the yield of *Pleurotus ostreatus*. Substrate optimization studies revealed the application of 4% humic acid for maximum yield. The nutrient value of the mushroom was found to be 40 g protein, 0.1 g fat and 19.5 g fibre. The optimum substrate composition even showed a drastic effect on the productivity and biological efficiency. The increased productivity and biological efficiency was found to be 18.47 % and 27.16 % respectively.

Table- 1a: Levels of variables used in the Central composite design with four independent variables for optimization and their significance

Variables	Range and levels				
	−2	**−1**	**0**	**+1**	**+2**
X_1:Conc.of bagasse(g/l)	17.5	21.25	25.0	28.75	32.50
X_2:Conc.ofwheat bran(g/l)	3.5	4.25	4.50	5.75	6.50
X_3:Conc. of urea(g/l)	3.0	4.0	5.0	6.0	7.0
X_4: Humic acid (%)	2.0	3.0	4.0	5.0	6.0

Table -1b: Central composite design matrix for optimization

Trials	Bagasse(g/l)	Wheat bran (g/l)	urea(g/l)	Humic acid (%)	Yield of Pleurotus *ostreatus*(g)	
	X_1	X_2	X_3	X_4	Experimental	Predicted
1	-1	-1	-1	-1	132	131.92
2	+1	-1	-1	-1	129	128.83
3	-1	+1	-1	-1	119	118.50
4	+1	+1	-1	-1	112	112.42
5	-1	-1	+1	-1	115	114.83
6	+1	-1	+1	-1	124	124.25
7	-1	+1	+1	-1	109	109.42
8	+1	+1	+1	-1	116	115.83
9	-1	-1	-1	+1	173	172.83
10	+1	-1	-1	+1	150	149.75
11	-1	+1	-1	+1	149	148.92
12	+1	+1	-1	+1	123	122.83
13	-1	-1	+1	+1	141	140.75
14	+1	-1	+1	+1	130	130.17
15	-1	+1	+1	+1	125	124.83
16	+1	+1	+1	+1	111	111.25
17	-2	0	0	0	113	113.42
18	2	0	0	0	97	96.75
19	0	-2	0	0	204	204.25
20	0	+2	0	0	172	171.92
21	0	0	-2	0	102	102.42
22	0	0	+2	0	74	73.75
23	0	0	0	-2	115	114.92
24	0	0	0	+2	151	151.25
25	0	0	0	0	124	124.33
26	0	0	0	0	125	124.33
27	0	0	0	0	123	124.33
28	0	0	0	0	125	124.33
29	0	0	0	0	124	124.33
30	0	0	0	0	125	124.33

Table -2a: Regression coefficients and their significance

Model term	Parameter estimate	Standard error	Computed t-value	P-value
Intercept	367.5486	14.25772	25.77892	7.76E-14
X_1	18.5	0.53029	34.88659	8.91E-16
X_2	-286.778	2.651448	-108.159	4.1E-23
X_3	64.29167	1.847796	34.79369	9.27E-16
X_4	61.16667	1.778863	34.38527	1.1E-15
X_1*X_1	-0.34222	0.007839	-43.6549	3.18E-17
X_2*X_2	28.33333	0.195982	144.5714	5.3E-25
X_3*X_3	-9.0625	0.11024	-82.2073	2.49E-21
X_4*X_4	2.1875	0.11024	19.84313	3.54E-12
X_1*X_2	-0.26667	0.05132	-5.19615	0.000109
X_1*X_3	0.833333	0.03849	21.65064	9.97E-13
X_1*X_4	-1.33333	0.03849	-34.641	9.89E-16
X_2*X_3	2.666667	0.19245	13.85641	5.91E-10
X_2*X_4	-3.5	0.19245	-18.1865	1.24E-11
X_3*X_4	-3.75	0.144338	-25.9808	6.92E-14

Table -2b: Regression Statistics and ANOVA for the regression model

Source	Degree of freedom	Sum of squares	Mean sum of squares	F	Significance F
Regression	14	17754.87	1268.205	3804.614	5.37E-24
Residual	15	5	0.3333		
Total	29	17759.87			

Multiple R 0.999859 ; R^2 0.999718 ; Adj R^2 0.999456 ; Standard Error 0.57735

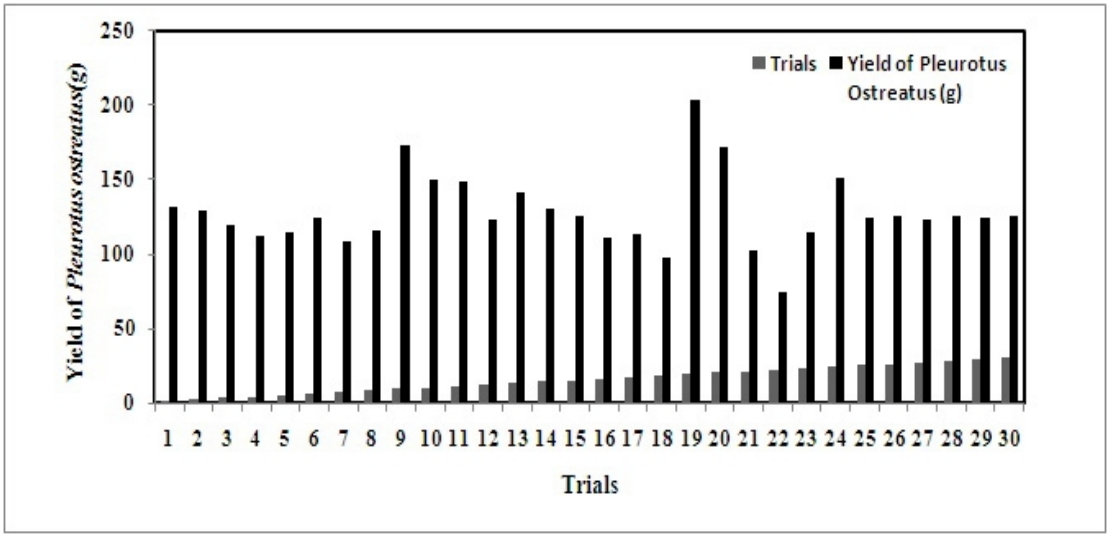


Table- 3: Protein, fat, Moisture, fibre, ash content in *Pleurotus ostreatus* and Productivity and Biological efficiency of substrates.

Trials	Protein (g)	Moisture (%)	Fat (g)	Fibre (g)	Ash (g)	M_F (g)	C_F (g)	C_D (g)	P (%)	BE (%)
1	22	79	0.7	2.3	4.9	132	1008	773	13.09	17.08
2	25	63	0.5	2.6	7.2	129	1009	775	12.70	16.65
3	29	73	0.9	18.6	3.2	119	1123	885	10.59	13.45
4	22	88	0.4	4.4	2.6	112	1158	884	9.67	12.68
5	21	82	0.9	3.3	3.1	115	1124	881	10.23	13.05
6	35	92	0.2	17.2	1.4	124	1135	880	10.92	14.09
7	18	95	0.8	2.9	2.5	109	1100	790	9.90	13.79
8	20	94	0.9	4.3	2.3	116	1105	780	10.49	14.87
9	21	89	0.4	5	3.2	173	1124	745	15.39	23.22
10	19	86	0.5	9.6	3.2	150	1120	769	13.39	19.51
11	22	93	0.9	7.9	2.6	149	1127	798	13.22	18.67
12	22	95	0.6	8.9	2.9	123	1121	783	10.97	15.71
13	25	96	0.4	11.0	4.9	141	1120	781	12.58	18.05
14	23	94	0.7	13.3	4.2	130	998	665	13.02	19.55
15	23	91	0.5	13.5	3.6	125	869	662	14.38	18.88
16	24	90	0.5	12.6	3.8	111	1001	783	11.08	14.18
17	32	89	0.6	4.6	3.4	113	1121	784	10.08	14.41
18	30	87	0.6	5.5	3.5	97	1124	754	8.62	12.86
19	40	82	0.1	19.5	3.1	204	1104	751	18.47	27.16
20	31	84	0.7	2.2	3.9	172	1127	854	15.26	20.14
21	29	91	0.8	3.5	4.1	102	1124	851	9.07	11.98
22	27	93	0.7	14.6	1.6	74	550	441	13.45	16.78
23	26	90	0.8	15.2	2.6	115	1125	952	10.22	12.08
24	20	97	0.5	7.9	2.6	151	1121	774	13.47	19.50
25	22	92	0.5	6.2	2.9	124	1112	752	11.15	16.49
26	23	74	0.4	5.9	2.8	125	1100	750	11.36	16.67
27	22	86	0.4	2.6	3.2	123	1132	852	10.86	14.44
28	25	82	0.3	5.2	4.2	125	1104	802	11.32	15.59
29	21	84	0.3	6.9	4.3	124	1105	802	11.22	15.46
30	26	91	0.3	5.2	4.2	125	1109	821	11.27	15.23

References

AOAC,1990. *Official methods of analysis of the Association of Official Analytical Chemists*, The Association:Arlington, VA. Vol. II, 15th ed. Sec.985:29.

Ahmad,I.1986.'Some studies on oyster mushroom (*Pleurotus sp*.) on waste material of corn industry',*M.Sc thesis. Department of plant Pathology, Faisalabad*, 50.

Brannon, C.A., and Somers, L.A.1985. 'Preparation and characterization of model humic polymers containing phosphorus'. *Soil Biology and Biochemistry,* 17:213-219.

Breene, W.M. 1990. 'Nutritional and medicinal value of specialty mushrooms'. *Journal of Food Protection*.53(10):883-894.

Bhak ,G., Lee, S., Hwang, S. and Song, M., 2005. 'Response surface analysis of solid state growth of *Pleurotus ostreatus* mycelia utilizing whey permeate'. *Biotechnology Letters*, Vol.27, pp.1537–1541.

Cohen, R., Hadar, Y. and Persky, L.,2002. 'Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus'*. *Applied Microbiology and Biotechnology*, 58:582-594.

Dundar, A., Acay, H., and Yildiz, A.2008. 'Yield performances and nutritional contents of three oyster mushroom species cultivated on wheat stalk'.*African Journal of Biotechnology*, 7 (19):3497–3501.

Draper, N.R., and Smith, H.1981. '*Applied regression analysis'*. New York, John Wiley and Sons, 141-183

Du, R.Q. 2003.'*Biological statistics'*. Beijing,Higher Education Press, 119-41.

Fasidi, I.O., and Kadiri, M. 1993. 'Effect of sporophore maturity on chemical composition of *Volvarielia esculenta* (Mass) Singer, a Nigerian edible muchroom'. *Die Nahrung*,37: 269-273.

Justo, M.B., Guzman, G.A., Mejia, E.G., and Diaz, C.L.G., Martinez, G.,Corona, E.B.1999. 'Calidad proteínica de tres cepas mexicanas de setas(*Pleurotus ostreatus*). Archivos Latinoamericanos de Nutricion', 49(1)81-85.

Jwanny, E.W., Rashad ,M.M., and Abdu, H.M. 1995. 'Solid state fermentation of agricultural wastes into food through *Pleurotus* cultivation'. *Biotechnology and Applied Biochemistry*,50, 71-78.

Kadiri, M., and Fasidi, I.O.1990.'Variation in chemical composition of *Chlorophyllum molybditis* (Mayerex Fr) Masses and *Pleurotus tuber-regium* (fries) during fruit body development'.*Nigerian journal of science*, 24:86-89

Kononova, M.M.1966. '*Soil organic matter. Its nature, its role in soil formation and in soil Fertility*'. Pergamon, Oxford, 2nd English edition, 544.

Manzi, P., Gambelli, L., Marconi, S., Pizzoferrato, L. and Vivanti, V., 1999. 'Nutrients in edible mushrooms an inter-species comparative study'. *Food Chemistry*,65:477-482.

Manzi, P.,Aguzzi, A., and Pizzoferrato, L.2001. 'Nutritional value of mushrooms widely consumed in Italy'. *Food Chemistry*,73:321-325.

Meire Cristina Nogueira de Andrade, João Kopytowski Filho, Leila Nakati Coutinho, Mário Barreto Figueiredo, and Marli Teixeira de Almeida Minhoni,2007. 'Productivity, biological efficiency, and number of *Agaricus blazei* mushrooms grown in compost in the presence of *trichoderma* sp. and *Chaetomium olivacearum* contaminants'. *Brazilian Journal of Microbiology*, 38:243-247.

Parent,G. and Thoen, D.1977. 'Food value of edible mushroom from Upper Shaba region'. *Economic Botany*,31: 436-445.

Quimio, T.H.1976. 'Cultivation Ganoderma the "*Pleurotus-way*" mushroom'. *Newsletter for Tropics*, 6:12-130

Quimio, T.H.1978.'Indoor cultivation of *Pleurotus ostreatus'*. *Philippines Agriculturist*, 61:253-262.

Ragini Bisaria, Mira Madan , Mukhopadhyay, S.N.1983. 'Production of biogas from residues from mushroom cultivation'. *Biotechnology Letters*, 5(12):811-812.

Ranzani, M.R., and Sturion, G.L.1998. 'Amino acid composition evaluation of *Pleurotus* sp. cultivated in banana leaves'.*Archivos latinoamericanos de nutricion*,, 48(4)339-348.

Solomko, E.F., and Eliseeva, G.S., 1988. 'Biosynthesis of vitamins B by the fungus *Pleurotus ostreatus* in a submerged culture'. *Prikl Biokhim Mikrobiol*, 24:164-169

Schnitzer, M.1978.'*Humic substances, chemistry and reactions'*. In: Soil Organic Matter. Elsevier Science Publishing Co., New York.

Schnitzer,M.1986. 'Binding of humic substances by soil mineral colloids. In: Interactions of Soil Minerals with Natural Organics and Microbes'. *Soil Science Society of America*, *Wisconsin*,83-87.

Stevenson, I.L., and Schnitzer, M.1982. 'Transmission electron microscopy of extracted fulvic and humic acids'. *Soil Science*,133:179-185.

Shah, Z.A., Ashraf, M. and Ishtiaq Ch, M. 2004. 'Comparative Study on cultivation and yield performance of Oyster mushroom (*Pleurotus ostreatus*) on Different Substrates (Wheat Straw,Leaves, Saw Dust)'.*Pakistan Journal of Nutrition*, 3(3):158-160.

Shah,H., Iqtidar, A.K., and Shagufta, J. 1997. 'Nutritional composition and protein quality of Pleurotus mushroom'. *Sarhad Journal of Agriculture* , 13(6): 621-626.

Tan, K.K.,1981. 'Cotton waste is a fungus (*Pleurotus)*good substrate for cultivation of *Pleurotus ostreatus*, the oyster mushroom'. *Mushroom Science* ,11:705-710.

Vaughan D, Linehan D J (1976) 'The growth of wheat plants in humic acid solutions under axenic conditions'.*Plant and Soil*,44:445-449.

Vinklarkova, K., and Sladky, Z. 1978. 'Exogenous Regulators in the Mycelium of *Pleurotus ostreatus* after Exogenous Application'.*Folia Microbiol*, 28: 55-59.

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Original Article

Biotechnological synthesis of gold nanoparticles of *Azadirachta indica* leaf extract

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Abstract

Development of eco-friendly process for the synthesis of nanoparticles is one of the main steps in the area of nanotechnology research. Here, we studied the Biological synthesis of gold nanoparticles using *Azadirachta indica* plant leaf extract .The synthesized nanoparticles are confirmed by color changes and it was characterized by UV-visible spectroscopy. The plant based route could be considered to be an environmental friendly or green biological method of nanoparticles production.

Key words: Gold nanoparticles, UV – visible spectroscopy, *Azadirachta indica*, HPLC.

Introduction

Nowadays, the preparation of nanoscaled gold materials has become very important due to their unique properties, which are different from those of the bulk materials (Armendariz *et al*.,2004). The properties of these particles in applications as diverse as catalysis, sensors and medicine depend critically on the size and composition of the nanoparticles (Haverkamp *et al*.,2007). Production of nanoparticles can be achieved through mainly three methods such as, Chemical, Physical and Biological methods. Since noble metal nanoparticles such as gold, silver and platinum nanoparticles are widely applied to human contacting areas, there is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals. Biological methods of nanoparticles synthesis using microorganism, enzyme, and plant or plant extract have been suggested as possible ecofriendly alternatives to chemical and physical methods (Song and Kim**,**2008). Specifically, the study has been attempted bioreduction of chloroaurate ions or silver ions by the broths of geranium and neem (Shiv Shankar *et al*.,2004a;Shankar *et al*.,2004b). Also gold nanotriangles synthesisized using Tamarind leaf extract and studied their potential application in vapour sensing (Ankamwar *et al*.,2005). Most recently, a study has been demonstrated synthesis of gold nanotriangles and silver NPs using *Aloe vera* plant extracts (Chandran *et al*.,2006). Already some works have been reported on synthesis of gold nanoparticles by *A. indica.* In the present study we have investigated biosynthesis of gold nanoparticles by *Azadirachta indica* and concluded with reason for this synthesis. We used this plant leaf extract since this plant is easily available in our university campus itself and also in all over Tamil Nadu, India

Materials and Methods

Collection and Extract Preparation

Collected the plant leaves from our university campus itself, and allowed to dry for 2 weeks at room temperature. The plant *A. indica* was used. Leaves were dried at room temperature. The plant leaf broth solution was prepared by taking 5g of thoroughly washed and finely cut leaves in a 300mL Erlenmeyer flask with 100mL of sterile distilled water and then boiling the mixture for 5 min. They were stored at 4^{o}C and used within a week.

Synthesis and Characterization

For the synthesis of Au- NPs (Gold nanoparticles), two boiling tubes were taken, one containing 10ml of 1*m*M Hydrogen tetra chloro aurate (Himedia, Mumbai) solution as control and the second flask containing 9ml of 1mM Hydrogen tetra chloro aurate solution and 1ml of plant leaf extracts as test solution were incubated at room temperature for 1-2 hours. The gold nanoparticle solution thus obtained was purified by repeated centrifugation at 15,000 rpm for 20 min. Supernatant is discarded and the pellet is dissolved in deionised water. The gold nanoparticules were confirmed by colour changes and qualitatively characterized by UV-visible spectrophotometer on a Perkin Elmer (Lamda 25)

Results and Discussion

The color change showed the presence of gold nanoparticles in the *Azadirachta indica* leaf extract and it was characterized by UV-Visible spectrophotometer and monitored by taking readings at regular time intervals in a Perkin Elmer (lambda25) UV–Visible spectrophotometer. The strong broad peak located at 550nm was observed for gold nanoparticles.

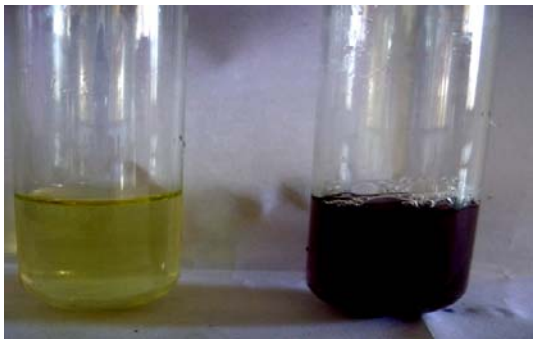

Fig.1: 1mM Hydrogen Tetra Chloro Aurate solution before adding extract (*Azadiracda indica*) (left), after adding extract to the solution

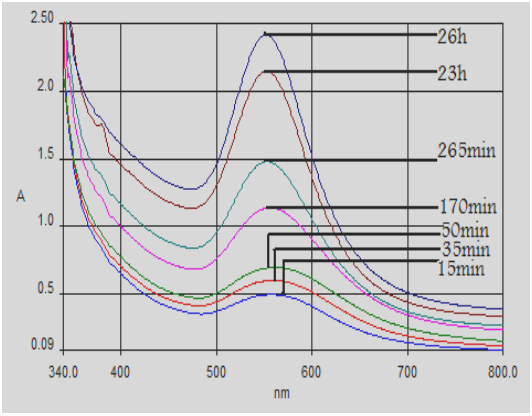

Fig. 2: Strong broad peak of gold nanoparticles *Azadirachta indica* leaf extract

The color change showed the presence of gold nanoparticles in the *A. indica* leaf extract and it was characterized by UV-Visible spectrophotometer and monitored by taking readings at regular time intervals in a Perkin Elmer (lambda25) UV–Visible spectrophotometer. The strong broad peak located at 550nm was observed for gold nanoparticles.

Gold nanoparticles were synthesized from Hydrogen tetra chloroaurate solution containing Au^{+} ions by treating with the leaf extract of Neem (*A. indica*). The supernatant of *A. indica* changed the Hydrogen tetra chloroaurate solution to a pink ruby red color within 15 minutes of reaction with the Au^{+} ions (as shown in Fig.1). The appearance of the pink ruby red color indicated the formation of gold nanoparticles.

The gold nanoparticles initially confirmed by color changes and it was characterized by UV- Visible spectrophotometer. This technique has proved to be very useful for the analysis of nanoparticles. The UV- visible spectra showed a strong Plasmon resonance which was centered approximately at 550 nm (as shown in Fig.2). Observation of this strong broad plasmon peak has been well documented for various Me- NPs, with sizes ranging all the way from 2 to 100 nm (Henglein,1993). Sadeghian *et al*.,(2007) analysed the *Azadirachta indica* plant leaf extracts bioactive compounds by HPLC (Table I).Compared to all the other compounds the major bioactive compounds are found as Salanin, Nimbin, Azadirone and Azadirachtins and with this we can conclude the result that it may be one of the reasons for the reduction of gold nanoparticles.

Conclusion

In conclusion, the gold nanoparticles were synthesized using plant leaf extracts of *Azadirachta indica* as this plant is easily available in our university campus and all over Tamil Nadu, India. Initially synthesis was confirmed by color changes and Characterized by UV-visible spectroscopy. According to Sadeghian *et al*.,(2007) analysis the major bioactive compounds are Salanin, Nimbin, Azadirone and Azadirachtins in the *Azadirachta indica* plant leaf extract. With this we can conclude that it may be one of the reasons for the reduction of gold nanoparticles.

References

Armendariz,V., *et al*.2004. Size controlled gold nanoparticle formation by *Avena sativa* biomass: use of plants in nanobiotechnology. *Journal of Nanoparticle Research*, 6: 377–382.

Haverkamp, R.G. *et al*.,2007. Pick your carats: nanoparticles of gold–silver–copper alloy produced *in vivo*. *Journal of Nanoparticle Research*, 9:697–700.

Song, J.Y. and Kim, B.S.2008. Rapid biological synthesis of silver nanoparticles using plant leaf extracts. Bioprocess Biosyst Eng.

Shiv Shankar, S, Rai A, Ankamwar B, Singh A, Ahmad, A, and Sastry, M.2004a. Biological synthesis of triangular gold nanoprisms. *Nat Mater*., 3:482–488

Shiv Shankar, S., Rai, A., Ahmad, A., and Sastry, M. 2004b. Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using neem (*Azadirachta indica*) leaf broth. *J. Colloid Interface Sci* .,275:496-502

Ankamwar; B., Chaudhary, M., and Sastry, M. 2005. Gold Nanotriangles biologically Synthesized using Tamarind leaf extract and Potential application in vapor sensing. *Synthesis and Reactivity in inorganic, metal-organic, and Nano-Metal Chemistry*, 35:19- 26.

Chandran S.P., Chaudhary, M., Pasricha, R., Ahmad, A. and Sastry, M.2006. Synthesis of Gold Nanotriangles and Silver Nanoparticles using *Aloe vera* plant extract. *Biotechnology Progress*,577 – 583.

Henglein, A. 1993. Physicochemical properties of small metal particles in solution: "microelectrode" reactions, chemisorption, composite metal particles, and the atom-to-metal transition. *J. Phys.Chem.*,5457–5471.

Sadeghian,M. M. and Azaienezhad, F.M. 2007. Investigation of compounds from *Azadirachta indica (Neem). Asian Journal of Plant sciences*, 6(2): 444-445.

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Original Article

Influence of media on protease production by *Beauveria bassiana* (Bals.) Vuil. and stability towards commercially available detergents, surfactants and enzyme inhibitors

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Abstract

The present study was undertaken to evaluate protease production by fungal biopesticide *Beauveria bassiana* (Bals.) Vuil. using casein media supplemented with bovine serum albumin, gelatin, antiserum, egg white, egg yolk, whole egg homogenate, fish meal and rice, wheat, green gram, black gram and Bengal gram extracts under shake flask condition. All the media supported growth and enzyme production...Horse gram extract supplemented casein media supported maximum protease production followed by wheat extract and the enzyme was partially purified by ammonium sulfate precipitation and dialysis. The purity of the enzyme was confirmed by appearance of sharp single band with the molecular weight of 66KD after SDS -PAGE of the enzyme. The enzyme activity was found to be maximum pH 8.0 and temperature 70^0C. Moreover the effect of various detergents such as Henko, Ariel, Surf Excel, Rin surfactant, tween80 and triton X and inhibitors EDTA $CaCl_2$, $MnSO_4$ was studied. The enzyme could retain its activity in all the tested detergents except Ariel. Surfactants Tween80 and trilonX, EDTA at all the concentrations did not cause distinct effect. Among the inhibitors, $MnSO_4$ at all the concentration, and $CaCl_2$ at 4 and 5*m*M completely inhibited protease activity.

Key words: protease; *Beauveria bassiana;* enzyme activity; detergents; inhibitors

Introduction

Proteases, also known as proteinases or proteolytic enzymes, are a large group of enzymes belong to the class of enzymes known as hydrolases., which catalyse the reaction of hydrolysis of various bonds with the participation of a water molecule (Urtz and Rice 2000). The current estimated value of the worldwide sales of industrial enzymes is $1 billion. Of the industrial enzymes, 75% are hydrolytic. Proteases represent one of the three largest groups of industrial enzymes and account for 60% of the total worldwide sale of enzymes (Cho and Cho,2004).

Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial applications particularly proteases are used in a variety of industrial applications as laundary detergents, pharmaceuticals, leather products, food products and even in waste processing industry **(**Dutta *et al*.,2005). Microbial proteases are the most widely exploited industrial enzymes with major applications in detergent formulations. Over the past 30years, the important of proteases in detergents has well recognized and various factors such as pH, ionic strength, temperature, detergent composition, bleach system and mechanical handling influencing protease activity has reported (Shuang *et al*.,2009). In the present study the various nutrient sources such as casein media supplied with antiserum, gelatin, bovine serum albumin, egg yolk, egg white, mixture of egg yolk and egg, white, rice ,wheat, Been gram, Bengal gram on protease production by *Beauveria bassiana* (Bals.) Vuil. under different temperature and pH and stability of the enzyme was also studied with commercially available detergents Henko, Ariel, Surfexcel, Rin and Tide, surfactant tween 80 and triton X and inhibitors EDTA, $CaCl_2$, and $MnSO_4$.

Materials and Methods

Fungal strain

Beauveria bassiana was isolated from dead caterpillar of *Spodoptera litura* collected from groundnut field by standard methods and maintained on potato dextrose agar slant as monosporic culture

Screening for Protease Production

Screening for protease production was done using the inoculum media supplemented with 2% casein as substrate. The media was sterilized by autoclaving and poured into sterile Petri plates and allowed to solidify. The fungal plug was taken from edge of 7 days old actively growing culture on the PDA plate using 8mm sterile cork borer and transferred to the casein media and incubated at 32^0C for 3-4 days. The plates were observed from a clear zone around the growing fungal plug.

Inoculum Preparation

100ml of inoculam media was prepared in 250ml of conical flask (Yeast extract 500mg, peptone 500mg, glucose 1g and casein 2g, distilled water 100ml, pH 7.0) and sterilized by autoclavig. The sterilized media was inoculated with 0.1ml of *B.bassiana* spore suspension at the spore concentration of $1.0X10^8$ spores/ml. The inoculated flask was incubated at 3^0C under shaking condition

Protease production

The inoculum media was prepared and sterilized as described earlier and supplemented with filter sterilized bovine serum albumin (1oomg) gelatin (100mg) 0.1 1% of egg white, yellow emulsion, both yellow and white 0.1%), fresh fish meal (0.1%). The fish meal was prepared by homogenization of fish tissue using phosphate buffer, filtered through muslin cloth and the collected filtrate was steam sterilized. The sterilized extracts were added into the inoculum media at 0.1% concentration.

Preparation of Cereal Extracts

The cereal extracts was prepared from rice, wheat, black gram and horse gram. The respective cereals was soaked in water for 1 hour and the soaked cereals were boiled in microwave oven (Samsung) and the boiled cereals were homogenized using domestic mixer and the slurry was diluted with water to get complete homogenous mixture. 100ml of this mixture was sterilized by autoclaving and used for protease production (10%). And inoculated with fungal spore suspension.

Enzyme Extraction and quantification Assay

The seeded flasks were incubated at 35^0C in shaker (The Science house) at 150 rpm for 3 days. After incubation the media was filtered through the cheese cloth and the collected filtrate was centrifuged at 10,000rpm for 15 minutes. The supernatant obtained was used as crude enzyme source.

Enzyme assay

The activity of protease was assessed in triplicates by measuring the release of trichloroacetic acid soluble peptides from 0.25 casein in 0.1%M Tris-Hcl buffer (pH8.0) at 60 C for 10 minutes. The reaction was terminated by the addition of 0.5ml of 15% trichloroacetic acid and then centrifuged at 20,000rpm for 5 minutes,after cooling.One unit of enzyme activity was defined as the amound of enzyme required to produce an increase in absorbance at 420nm equal to 1.0 in 60 minutes.

Purification

The supernatant obtained was concentrated by ammonium sulfate precipitation with different concentration of ammonium sulfate at 20,40,60 and 80%. The precipitate was dissolved and dialysed overnight against 10mM Tris HCL buffer at pH8.0 and the dialyzed product was lyophilized and 20mg of the lyophilized material was applied to a Bio-Gel P-60,60,000 molecular weight exclusion column (Bio-Rad laboratories). The elution buffer was Tris- HCL0.05M,0.1M KCL (pH 7)All fractions showing proteolytic activity were pooled and this purified protease was lyophilized.

SDS- PAGE

SDS- PAGE was carried to assess the molecular weight of protease.. The gel was polymerized from a mixture of 17.5 ml of 30% acrylamide -0.8% methylene bis acrylamide-17.5ml of 1.5M Tris hydrochloride (pH 8.8) – 35 ml of distilled water 35±l of *N,N,N',N'* tetra methylene diamine-0.70ml of ammonium per sulfate (75mg/ml). Electrophoresis was performed at 1^0C with 0.05*M* Tris-glycine buffer (pH 8.3). The protein band was visualized after staining with Commasive brilliant blue followed by destaining with methanol and acetic acid. The molecular weight of the band was identified using standard molecular markers.

Protein determination

The quantification of protein was determined by Lowry's method with BSA standard.

Effect of pH and Temperature on Protease activity

The lyophilized enzyme was incubated with 2ml of phosphate buffer with different pH values ranged from 5.0, 6.0, 7.0, 8.0 and 9.0 incubated at 30^0C for 1hour. The enzyme assay was done as described earlier. The effect of temperature was studied at at different temperatures 40,50, 60, 70 and 80^0C respectively for 1 hour.

Effect of detergents, surfactants and inhibitors on enzyme activity

The detergents used in the study were Henko,Ariel, Surfexcel, and Rin and diluted. In double distilled water to a final concentration of 5,10,25,50 and 100m g/ml.A protease concentration of 0.55 mg/ml was incubated with respective concentration of detergents,1,2,3,4 and 5mM concentrations of EDTA,Cacl2 and Mnso4 and surfactants tween 80 and triton X (1,2,3,4 and 5%) was added and mixed well. All the tubes were incubated at 40^oC for 1hour. Untreated control was also maintained. After the incubation period, the enzyme quantification was carried out at respective treatment as mentioned earlier.

Results and Discussion

When *B.bassiana* grown in different media,proteolytic activity appeared in all the tested media Among the different media, casein media supplemented with horse gram extract (2.24 Units/ml) supported maximum enzyme production followed by wheat extract (1.99 units/ml). Casein media supplemented with bovine serum albumin and antiserum recorded 1.91 and 1.51U/ml respectively. Followed by antiserum and BSA supplemented media, egg yolk showed 1.44U/ml. Casein media with remaining nutrient sources also showed protease activity. Casein media supplemented with fish meal (0.83U/ml) gelatin (0.85U/ml) and egg white 0.98U/ml), rice extract (1.23U/ml), black gram extract (1.99U/ml)was recorded (Table- 1).

Table-1. Influence of media on protease production by *Beauveria bassiana*

Sl. No	Media	Enzyme activity (U/ml)
1	Casein media	1.07
2	Casein media + bovine serum albumin	1.91
3	Casein media + antiserum	1.51
4	Casein media + gelatin	0.85
5	Casein media + egg white	0.98
6	Casein media + egg yolk	1.44
7	Casein media +whole egg	1.25
8	Casein media + fish meal	0.83
9	Casein media + rice extract	1.23
10	Casein media + wheat extract	1.99 *
11	Casein media + Horse gram extract	2.24 *
12	Casein media + black gram extract	1.99

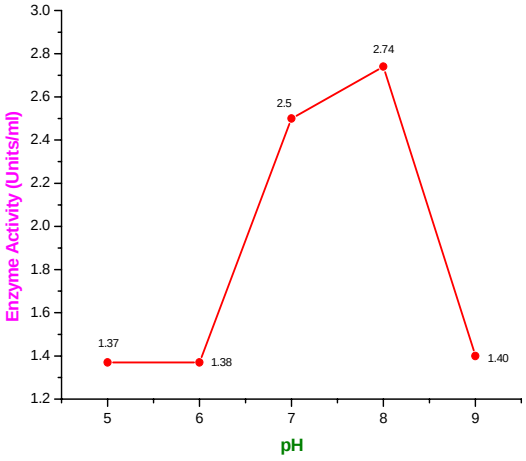
*Significant at (P>0.05) level

Ammonium sulfate precipitation of filtered culture supernatant showed that protease fractioniated at 60% saturation and the fractions were further purified by GelB and the purity of the enzyme was detected by appearance of single protein band detected by SDS- PAGE with molecular weight of 66KD (Fig.2) Ariane *et al*.,2009 and Eliana *et al*.,(2008) studied protease production by B. bassiana using cuticle of *Rhammatocerus Schistocercoides* and *Hypothenemus hampei.*The protease was active in a broad range of pH from 5.0 to 9.0with the optimum pH 8.0 The optimum temperature for this protease activity was70◦C (Fig1, a-b,).

Stability of protease with commercially available detergents, surfactants and inhibitors

Protease from *B. bassiana* retained more than 90% of its activity in all the tested concentration of detergents except Ariel. The enzyme activity at respective concentration of Henko was 2.24,2.24,2.19,2.19 and 2.19, 2.22,2.22,2.18,2.18 and 2.18U/ml in Surf excel. In the case of Rin, 2.19,2.19,2.19,2.17 and 2.17U/ml was recorded. Enzyme activity was not recorded in all the tested concentrations of Ariel except 5mg concentration. 0,99U/ml was recorded in this concentration (Table- 2).

(**a**)



(**b**)

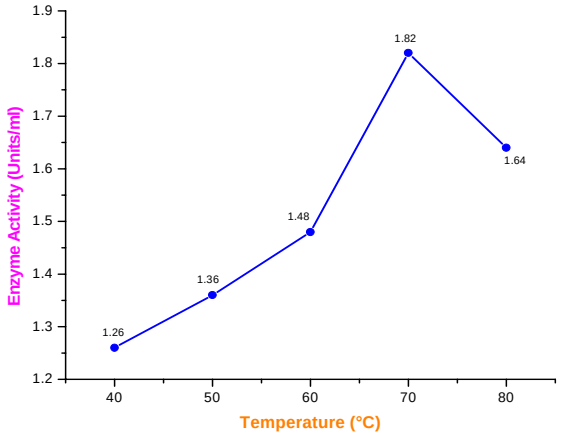


Fig.1: Effect of pH (a) and temperature (b) on protease activity

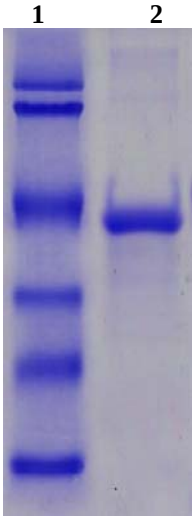


Fig. 2: SDD-PAGE electrogram of Protease
1.Protein marker;
2.Purified protease with 66 KD

No inhibitory activity was observed in all the tested concentration of EDTA. Complete enzyme inhibition was recorded at all the tested concentrations of $MnSO_4$ and $CaCl_2$ at 4 and 5mM concentration. There was no distinct reduction on enzyme activity was recorded in Tween 80 and Trilon X. Nascimento and Martins,(2006) studied stability of protease produced by soil isolate of *Bacillus* sp.with commercially available detergents and they observed protease could retained more than 80% of its activity in all the tested concentrations of detergents. Banerjee *et al*., (1999) also evaluated stability of protease from *Bacillus brevis* with the commercially available detergents and the activity was also not affected .Based on these findings, *B.bassiana* produces protease in cheap sources and the protease produced by *B.bassiana* shows best compatibility with detergents which clearly reveals application of the enzyme as biodetergents for applications in laundry detergents.

Table -2: Effect of detergents, surfactants and inhibitors on protease activity (U/ml)

S.No	Treatment	Concentration	Enzyme activity (U/ml)
1	Detergents		
1a	Henko	5	2.24
1b	Henko	10	2.24
1c	Henko	25	2.19
1d	Henko	50	2.19
1e	Henko	100	2.19
2a	Surf excel	5	2.22
2b	Surf excel	10	2.22
2c	Surf excel	25	2.18
2d	Surf excel	50	2.18
2e	Surf excel	100	2.18
3a	Rin	5	2.19
3b	Rin	10	2.19
3c	Rin	25	2.19
3d	Rin	50	2.17
3e	Rin	100	2.17
4a	Ariel	5	0.99
4b	Ariel	10	0.00
4c	Ariel	25	0.00
4d	Ariel	50	0.00
4e	Ariel	100	0.00
5	Surfactants		
5a	Tween 80	1%	2.21
5b	Tween 80	2%	2.21
5c	Tween 80	3%	2.21
5d	Tween 80	4%	2.21
5e	Tween 80	5%	2.21
6a	Triton X	1%	2.20
6b	Triton X	2%	2.20
6c	Triton X	3%	2.20
6d	Triton X	4%	2.20
6e	Triton X	5%	2.20
7	Inhibitors		
7a	EDTA	1mM	2.20
7b	EDTA	2mM	2.20
7c	EDTA	3mM	2.20
7d	EDTA	4mM	2.20
7e	EDTA	5mM	2.20
8a	$MnSO_4$	1mM	0.00
8b	$MnSO_4$	2mM	0.00
8c	$MnSO_4$	3mM	0.00
8d	$MnSO_4$	4mM	0.00
8e	$MnSO_4$	5mM	0.00
9a	$CaCL_2$	1mM	1.61
9b	$CaCL_2$	2mM	1.23
9c	$CaCL_2$	3mM	1.01
9d	$CaCL_2$	4mM	0.00
9e	$CaCL_2$	5mM	0.00
10	Control		2.24

References

Cho, H.Y. and Cho, N. 2004. Optimization of submerged culture conditions for protease production and its enzymatic properties. *Journal of the Korean Wood Science and Technology,* 32: 12-19.

Dutta, J.R., Dutta, P.K. and Banerjee,R. 2005. Modeling and optimization of protease production by a newly isolated *Pseudomonas sp*. using a genetic algorithm. *Process Biochemistry,* 40: 879-884.

Shuang, L, Bingfang, H., Zhongzhong, B. and Pingkai, O. 2009. A novel organic solvent stable alkaline protease from organic solvent–tolerant. *Journal of Molecular Catalysis,* 56: 85–88

Ariane, C., Donatti Maria, H., Fungaro P. Marcia, C. and Furlaneto, 2008. Production and regulation of cuticle degrading proteases from *Beauveria bassiana* in the presence of *Rhammatocerus schistocercoides* cuticle. *Current Microbiology,* 56:256-260.

Eliana Tiemi Ito, Geni Varéa Pereira, Dalva Tomoe Miyagui, Maria Helena Pimenta Pinotti and Pedro Manoel Oliveira Janeiro Neves 2007. Production of extra cellular protease by a Brazilian strain of *Beauveria bassiana* reactivated on coffee berry b*orer Hypothenemus hampei. Brazilian Archives of Biology and Technology,* 50:217-223.

Nascimento and Martins,2006. Studies on stability of protease from *Bacillus* sp. compatability with commercial detergent. *Brazilian Journal of Microbiology,* 37-311.

Banerjee, G.C., Sari, R.K Azmi, W. and Soni, R.1999. Thermostable protcteease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochemistry*,35: 213-219.

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Original Article

Evaluation of amino acids profiles in human saliva using Reverse phase high performance liquid chromatography

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Abstract

The present investigation was carried out to analyses the possible changes of salivary amino acids during various phases of menstrual cycle. To detect the ovulatory day we used salivary ferning test on a microscope slide to form fern like crystals when it dries which shows the estrogen and crystals like sodium and potassium changes there shape for form a crystal like structure to note the day of LH surge during the day of ovulation will produced. The present study is aimed at knowing the presence and amount of amino acids in the saliva of human female at the age of varies reproductive state. Agilent high Performance Liquid Chromatography (HPLC) system was used to analyze the level of amino acids during the ovulatory and luteal phase in human saliva followed by derivatization method. Ethanolamine and aspartic acid are the amino acids uniquely present during the ovulatory phase of the cycle. Further, the amount of arginine (225.36±50.36) is significantly higher than that of other amino acids during ovulatory phase (13th day). The method of estimating clinically important amino acids in saliva may be exceedingly accurate result for ovulation prediction and to avoid the fertility rate. Detailed knowledge of the possible variations in human saliva is very important because we can develop a noninvasive diagnostic kit by studying the nature of the amino acids.

Key words: Amino acids, Lutenising hormone, Saliva, menstrual cycle

Introduction

The exact time of ovulation is important because it would help to identify the fertile period and thereby give treatment in fertile therapy. The prediction of ovulation period still remains a challenge for the investigators. Human saliva an easily available biological fluid, which shows cyclic variation in its composition during the menstrual cycle (Tandra and Bhattacharaya, 1989). Historically, salivary analyses of female sex hormones were used for fertility monitoring (Hofman,2001). However, recent findings indicate that these assays may be useful beyond the study of reproductive concerns. Sex steroid hormones appear to play a significant role in the physiology of the human oral cavity. A number of studies suggest that oral soft tissues are sensitive to changes in female sex steroid blood levels. Ovulation is a part of the menstrual cycle. This is caused by the complex ad interrelated of various hormones. During the process, the ovary and some of the epithelial cells become separated and surrounded with connective tissue. Then the follicle moves nearer the surface as the ovum nears maturity and rupture to extrude the ovum to the surface of the ovary (Lyons et al., 1989). In the physically mature female these are usually one egg released from the ovary about every 28 days. The release of egg from ovary was denied ovulation. The detection of ovulation is one the major problem in female reproduction in humans (Tandra and Bhattacharaya, 1989). Many test and techniques have been used to confirm ovulation in women wanting to become pregnant women. There are some direct methods available in the market such as laparoscopy and high-resolution transvaginal ultrasound examination, which are too invasive for this is repeated or routine use. To follow the noninvasive test, salivary ferning test is better for the earlier detection of LH spike in menstrual cycle formation of fern like formation during the period of ovulation (Alagendran et al., 2007,

Craft and Peters, 1971). Importantly, the identification of estrogen accessibility in these tissues has significant clinical importance and suggests a direct role for estrogen in the physiology of oral mucosa and salivary gland function. The present study is aimed at knowing the presence and amount of amino acids in the saliva of human female of vary in different reproductive state which could be used as the biological tool of earlier detection of ovulation.

Materials and Methods

Sample collection

The saliva was collected using Salivette spitting method (Sardeste, Germany) from 20 different female volunteers during the age (16-30 years) having normal menstrual cycle (Navazesh,1993). The samples were collected during follicular, ovulatory and luteal phases with help of the day of menses. The samples were screened through nylon mesh (16-120 µm) at the time of collection and were stored at -20^{o}C for further use.

Salivary ferning test

Taking a drop of sample of saliva, placing it on a microscope slide and allowing it to dry, and see if it forms a distinct 'ferning' pattern. The ferning pattern was believed to be caused by changes in estrogen levels and certain minerals in the cervical mucus at ovulation. However there is also a change in content of hormones and minerals in saliva around the time of ovulation, which cause the saliva to from fern like crystals when it dries. A miniature microscope uses this phenomenon, which is a useful aid for the detection of ovulation (Alagendran *et al*., 2007).

Analysis of amino acids by high performance liquid chromatography

Amino acid separation reagents

Sodium phosphate monobasic monohydrate, sodium hydroxide, boric acid, acetonitrile (LC grade), and methanol (LC grade) were obtained from Merck (Darmstadt, Germany). OPA reagent was prepared as described (Agilent art. 5061-3335, Palo Alto, CA). Borate buffer was prepared by adjusting 0.4 N boric acid to pH 10.2 with NaOH. Constant-boiling HCl was obtained from Sigma-Aldrich (St. Louis, MO). Chromatographic-grade water was produced by a Milli pore system (Millipore, USA)

Preparation of Amino acids

The saliva samples were collected in falcon tubes and mixed with 250 µl sulphosalicyclic acid centrifuged for 4000 rpm for 8 mins at 4°c, the supernatant was filtered with milli.Q syringe size (0.22 µm) is used for further analysis. Protein samples (10–60 µg) were transferred into the glass test tubes and spiked with 0.5 mM norvaline. They were quickly spun in a low-velocity centrifuge, then frozen and dried in a lyophilizer. Samples were then transferred into the reaction vial containing 0.5 mL of constant-boiling HCl on the bottom. Up to 12 test tubes could be accommodated in a reaction vial. The reaction vial was tightly closed and transferred into a pre-heated oven at 110°C for 18 h. The reaction vial was cooled at room temperature, then carefully opened under an aspirated hood. The test tubes were centrifuged and dried again in the lyophilizer to remove any liquid traces (condensed vapors). The dried residues were dissolved in 100 µL of 0.1 N HCl and transferred into the HPLC glass insert vials (Reason, 2003)

Instrumentation

Analyses were performed using an Agilent 1100 Liquid Chromatograph, equipped with a binary pump delivery system (G1312A), robotic autosampler (G1313A), column thermostat (G1316A) and multi-wavelength detector (G1365A).

Derivaitization Procedure

Chromatography conditions were in accordance with the Agilent HPLC method. Briefly, the hydrolyzed samples and the norvaline-spiked amino acid standard solutions were automatically derivatized with OPA by programming the robotic autosampler (Table 1). After derivatization, an amount equivalent to 2.5 µL of each sample was injected on a Zorbax Eclipse-AAA column, 5 µm, 150 × 4.6 mm (Agilent), at 40°C, with detection at λ = 338 nm. Mobile phase A was 40 mM NaH2PO4, adjusted to pH 7.8 with NaOH, while mobile phase B was acetonitrile/methanol/water (45/45/10 v/v/v). The separation was obtained at a flow rate of 2 mL/min with a gradient program that allowed for 1.9 min at 0% B followed by a 16.3-min step that raised eluent B to 53%. Then washing at 100% B and equilibration at 0% B was performed in a total analysis time of 26 min. Amino acid concentrations were calculated using the determination and peak areas relative to the area of the internal standard (Reason, 2003;

Gnanou et al., 2004). Experiments to establish the recovery for amino acids mixture through different phases including precipitation step was carried out through the procedure and the percent of recovery was assessed (Henderson et al., 1999).

Statistical analysis

Values are expressed in Mean ± SE. Those means in the same vertical column that are not marked with the same superscript letters are significantly different at P≤ 0.05 and 0.01 level (Duncan's multiple range test) assessed by One way ANOVA (SPSS Software, USA).

Results and Discussion

Table 1 gives the amount of aminoacids present in the women saliva of various phases like follicular (6-12 days), ovulatory (13-14 days and luteal phase (15-28 days) during the menstrual cycle. The amount varied from each particular amino acid of different phases. The level of amino acids in the ovulatory phase is significantly higher than the follicular and luteal phases. Among the various age groups of women saliva the amount of amino acids present randomly high like aspartic acid, arginine and serine in the peak during the ovulatory phase.

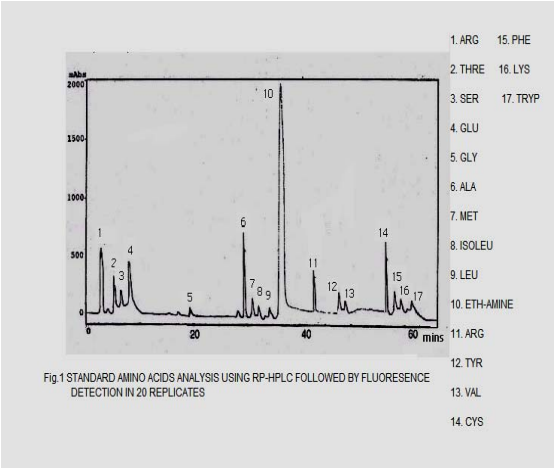
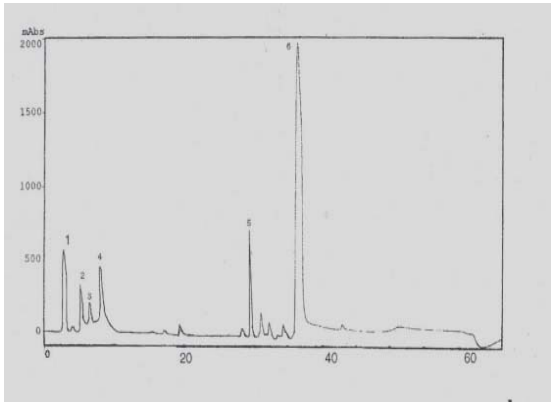
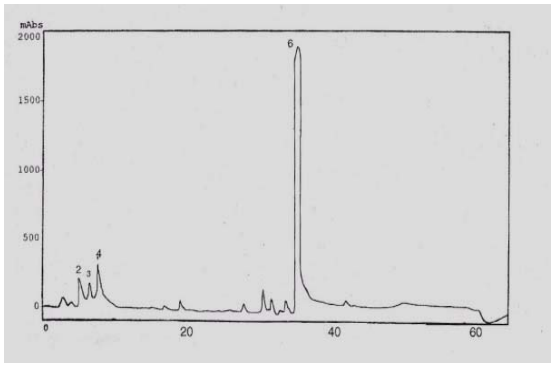


Fig.1: Standard HPLC analysis of salivary amino acids in human female subjects (n=20**)**



1.Aspartic acid, 2.Threonine, 3.Serine,4.Glutamic acid, 5. Ethanol amine 6. Arginine

Fig.2: HPLC analysis of salivary amino acids in the ovulatory phase of human female



2. Threonine, 3. Serine, 4. Glutamic acid, 6. Arginine

Fig.3: HPLC Analysis of Salivary amino acids in the luteal phase of human female

The high performance liquid chromatography (HPLC) analysis of amino acids during the 13th and 21st day of the menstrual cycle showed the presence of various amino acids (Fig. 1, 2 & 3). Six different amino acids viz Aspartic, Threonine, Serine, Glutamic acid Ethanolamine and arginine are present in different quantities during the 13th day (Fig. 2), where as only four amino acids are present in the 21st day (Fig. 3) of the menstrual cycle. Ethanolamine and aspartic acid are the amino acids uniquely present during the 13th day of the cycle. Further the amount of arginine is significantly higher than that of other amino acids during the 13th day. Next to arginine the concentration of aspartic acid and glutamic acid are higher in the same day. Considering each single amino acid, the recovery was found within the range 97–108%, while the average recovery, considering all the amino acids, was 100% (Table -1), which is largely within the 90–120% range that is considered acceptable.

Discussion

In 1985, the World Health Organisation (WHO) converts a task force to focus research efforts on ovulation detection (Spieler and Collins, 2001). By the late 1970's their role was redefined to focus on the determination of the fertile period. Proper education is necessary for appropriate use of ovulation prediction in home. At present a variety of tests are available, and the simplicity of the process varies with each product. In the present study an attempt was made to detect specific amino acids in saliva during ovulatory period. So that it can be used as an indicator of detecting ovulation (Fehring and Schlaff, 1998).

Table -1: Amount of amino acids present in the saliva of various stages of menstrual cycle (N=25).

Amino acids	Follicular phase (6-12 days)	Ovulatory phase (13-14 days)	Luteal phase (15-26 days)	*P*-Value
Aspartic acid	7.77±3.25	156.25±34.36	6.48±2.36	NS
Threonine	68.25±15.36	87.25±22.36	55.26±10.25	NS
Serine	85.25±20.5	120.35±26.5	68.36±14.25	0.01
Glutamic acid	40.25±13.56	132.69±16.3	39.25±11.25	NS
Glycine	12.36±23.58	46.98±6.58	109.58±14.36	0.01
Alanine	65.39±11.36	85.36±16.35	63.12±9.98	0.01
Methionine	12.36±5.69	14.36±6.35	11.36±6.25	NS
Isoleucine	33.25±10.25	40.26±13.25	31.26±9.36	NS
Leucine	52.36±13.25	66.35±16.35	50.36±11.36	NS
Ethanolamine	9.36±2.15	12.36±6.35	8.23±1.32	NS
Arginine	123.5±20.6	225.36±50.36	120.25±15.36	0.05
Tyrosine	32.65±11.36	47.36±15.6	31.65±9.25	0.02
Valine	9.56±2.25	15.36±6.25	8.36±1.99	NS
Cysteine	15.36±3.68	22.36±7.69	14.33±2.10	NS
Phenylalanine	22.52±10.36	26.35±12.36	20.14±9.47	0.03
Lysine	30.25±15.36	39.15±20.26	29.58±12.22	0.015
Tryptophan	35.6±14.36	42.36±20.6	33.25±11.36	0.02

Values are Mean ± SE, NS: not significant

The present investigation revealed that the levels of amino acids in saliva were significantly higher during ovulation than that of follicular and luteal phase. Further the increase in amino acids concentration was noted in young aged women (21-25). These findings suggest that certain amino acids may be present in higher concentration in saliva. Several workers (Wharton and Patton, 1953, Steele et al., 1950) have reported urinary amino acid data for normal men and women as influenced by special diets and little correlation was shown between quantitative ingestion of protein and amino acids excretion sheft and Oldham (1952) and Miller et al., (1954), from their work revealed that the amino acid showed higher excretion during pregnancy. Similarly the present results provide new evidence that the amino acid levels are specifically excreted in high concentration during ovulation.

The present study shows that the amino acids content in the saliva during the period of menstruation possess a progressive metabolic response that accompanies the transition from the fluctuations of hormone during the period of ovulation surge (Spieler and Collins, 2001). Endogenous levels of estradiol, progesterone and other hormones are periodic throughout the menstrual cycle. In addition to variation in the underlying hormonal patters between and within women, there are considerable variations in menstrual cycle length (Miller et al., 1954, Munster et al., 1992) have found that the human salivary glands are shown to be the target organs of estrogen action. As noted in the present studies, the amino acids are excreted in higher concentration are due to the estrogen action on salivary glands which may have intern increase the amino acid levels during ovulation. Similarly considerable variation in the salivary components was observed during the menstrual cycle of the women, where an increase in the activities of salivary components tended to coincide with the increase of estrogen level. Thus the present study provides additional support for excretion of salivary components with estrogen peak (Cockle and Harkness,1978).

These results are in agreement with previous studies of amino acid analysis clearly indicated that there are three amino acids namely aspartic acid, glutamic acid and arginine are present in the ovulatory saliva while compare to follicular and luteal periods. Among these three amino acids aspartic acid appeared only during ovulatory phase. Such fluctuation may prove to be clinically important. While on HPLC analysis serine, glutamic acid, ethanolamine and arginine

are estrogenic dependent. Among these amino acids, threonine was reported in the urine of pregnancy women (Tenovuo et al., 1981). Further aspartic acid, ethanol amine, threonine, serine and arginine are more specific and showed the higher concentration in saliva when compared to other amino acids during the period of ovulation.

Salivary amino acids have provided to be a useful marker for the detection of ovulation during the menstrual cycle of women (Miller et al., 1954; Fehring and Schlaff, 1998). The tremendous physiological forces set in motion by conception are manifested by several biochemical changes closely allied to amino acid excretion (Tovar and Torres, 1992). The present investigation convincingly reported that the amino acids could be considered as ovulation indicating components. Up to now, accurate effective and economical methods are not available to detect the ovulation. The discovery of ovulatory related components offers a promising approach to develop a simple non-invasive method to predict the ovulation very effectively (Yen et al., 1972). Diagnostic agents for ovulation enable women to manage the family planning process up to the point where they need to seek professional assistance. These agents are convenient for women to perform at home and may allow for an improved chance of conception. Such test only saliva onto a test stick with results available in minutes, while other requires complication steps and some are painful. Indeed, the home ovulation detection tests enable people to take an active role in avoiding IVF test and detection of conception rate concomitantly. Advantages of these tests include convenience, decreased healthcare costs and early family planning.

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References

Alagendran S., Archunan G., and Achiraman S., 2007. Prediction of Ovulation in Women through the Occurrence of Salivary Fern Prototype. *The Icfai Journal of Life Sciences*, Vol. 1, No. 2, pp. 7-15.

Cockle S.K. and Harkness R.A. 1978. Changes in salivary peroxidase and polymorphonuclear neutrophil leucocyte enzyme activities during the menstrual cycle. *Bri. J. Obstet. Gynecol*., 85: 776- 782.

Craft I.L, and Peters T.J. 1971. Quantitative changes in plasma amino acids induced by oral contraceptives. *Clin. Sci*., 41: 301-307.

Fehring R., and Schlaff W. 1998. Accuracy of the ovulation fertility monitor to predict and detect ovulation. *Journal of Nurse Midwife*, 19: 233-237.

Gnanou J.V., Srinivas S.K., and Kurpad A.V. 2004. Automated derivatization wirh o-pthaldehyde for the estimation of amino acids in plasma using reverse phase high performance liquid chromatography. Indian. *J. Biochem. Biophys*., 41: 322-325.

Henderson J.W., Ricker R.D., Bidlingmeyer B.A., and Woodward C. 1999. Agilent Technical Note; 5980– 1193E.

Hofman L. 2001. Human saliva as a diagnostic specimen. *J. Nutr*., 131: 1621S-1625S.

Lyons P.M., Truswell S.A., Michael M., Jeanetta V., and Suzanne F.A. 1989. Reduction of food intake in the ovulatory phase of the menstrual cycle. *Am. J. Clin .Nutr*., 49: 1164-1168.

Miller S., Ruttinoebandi V., and Macy G., 1954. Urinary excretion of ten amino acids by women during the reproductive cycle. *Ibid*. 209: 795.

Munster K, Schmidi L, and Helm P. 1992. Length and variation in the menstrual cycle a cross sectional study from a Danish country. *British J. Obset. Gynecol*., 99: 422-429.

Navazesh M .1993. Methods for collecting saliva. Ann New York, Acad. Sci. 693: 73-77.

Reason A.J. 2003. Validation of amino acid analysis methods. In Smith BJ (ed), Protein Sequencing Protocols, 2nd Edition: Methods in Molecular Biology, Vol. 211. Totowa, NJ: Humana Press, 181–194.

Sheft B. and Andh Oldham B., 1952. Amino acid intakes and excretions during pregnancy. *J. Am. Dietet. Assoc*., 38: 313.

Spieler J.M. and Collins W.P. 2001. Potential fertility defining the window of opportunity. *J. Int. Med. Res.,* 29 (suppl 1): 3A-13A.

Steele, B. F., Reynolds M. S. and Baumann C. A., 1950. Amino acids in the blood and urine of human subjects ingesting different amounts of the same proteins. *J. Nutrition*, 40: 145.

Tandra P., and Bhattacharaya A., 1989. Cyclic changes in salivary lactate dehydrogenase, peroxidase and leucine aminopepidase during menstrual cycle. *J. Exp. Biol*., 27: 695-698.

Tenovuo J., Laine M., Söderling E., and Irjala K. 1981. Evaluation of salivary markers during the menstrual cycle: peroxidase, protein,and electrolytes. *Biochemical Medicine,* 25 337–345.

Tovar A. and Torres N. 1992. Amino acid transport: regulation, characterization and factors affecting the amino acid transport systems. *Rev. Inv. Clin*., 44:427-439.

Wharton, M. A., and Patton M. B., 1953. Amino acid excretion on different protein intakes. *J. Am. Dietet. Assoc*., 39: 762-764.

Yen S.S.C., Tsai C.C., Naftolin F., Vandenberg G., Ajabor L. 1972. Pulsatile patterns of gonadotrophin release in subjects with and without ovarian function. *J. clin. Endocrinol. Metab*., 34: 671-675.

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Original Article

In vitro direct regeneration of nodal explant of *Justicia prostrata* Gamble

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Abstract

An efficient protocol for plant regeneration through direct organogenesis from nodal explant of *Justicia prostrata* Gamble has been developed. Different concentrations (0.2, 0.5, 1.0, 1.5, 2.0 mg L^{-1}) of plant growth hormones (2, 4-D, BAP, NAA and IAA) were used to obtain optimal regeneration response. The highest rate of shoot multiplication was obtained in MS medium containing 0.5 and 1.5 of mg L^{-1} BAP. The maximum number of roots was produced on the medium containing 0.5 mg L^{-1} of NAA and BAP within 45 days duration. The tissue culture raised plants exhibited normal growth and *in vitro* flowering. The plantlets, thus developed were hardened and successfully established in soil with 90% survival rate. This protocol could be useful for the production of mass cultivation of *J. prostrata* Gamble.

Keywords: *Justicia prostrata;* direct organogenesis; BAP; *in vitro* flowering

Introduction

Justicia prostrata Gamble (Acanthaceae) is a small herb, which is widely distributed in the mountains of Western Himalayas and in southern parts of India. In the traditional system of medicine the hot water extract of the whole plant of *J. prostrata* is used as an antidepressant. The literature survey revealed that the petroleum ether extract of the aerial parts of the plant was found to possess four arylnaphthalide lignans, prostalidins A, B, C and retrochinensin (Ghosal *et al*., 1979). Justicidin-E and two more unknown arylnaphthalide lignans were isolated from petroleum ether extract of whole plant of *J. prostrata* (Sanmugapriya,2001). Although the plant contains various phytoconstituents, it is not evaluated pharmacologically to a greater extent other than antidepressant (Ghosal *et al*., 1979), anti-nociceptive (Sanmugapriya *et al*., 2003) and anti-ulcerogenic activities (Sanmugapriya *et al*., 2005). This paper describes an efficient and rapid direct regeneration method and *in vitro* flowering of *J. prostrata* using nodal explants.

Materials and Methods

Plant collection and explants sterilization

The fresh plant of *J. prostrata* was collected from St. Joseph's college campus, Tiruchirapalli and authenticated by Dr.Soosai Raj, Department of Plant Biology and Plant Biotechnology of St. Joseph's college. In the present study, the nodal part was used as explants. The nodal explants were washed thoroughly under running tap water for 10 min followed by treatment with a solution of 1% Bavistin for 3 minutes and 2% (v/v) of Teepol (Reckitt Benckiser, India) for 3 minutes and materials were washed in tap water for 3 min. Then the explants were thoroughly rinsed with sterile distilled water for 3 min. After that the explants were treated with 0.1% Mercuric chloride for 3 min and finally rinsed with autoclaved distilled water for 5 -7 times to remove the surface sterilant.

Culture conditions

The culture medium consisted of MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 3%(w/v) sucrose and solidified with 0.8%(w/v) agar. The pH of media was adjusted to 5.7 before autoclaving for 15 min at 121°C. The pH of the medium was adjusted with 0.1N NaOH or 0.1N HCl. Cultures were maintained in a growth chamber at 28°C±2°C with 16 h light/8 h darkness. The explants were excised with the help of sterile forceps and surgical blade. The nodal explants were cut into 0.5 -1.0 cm sized segments. The explants were cultured on different

concentrations (0.2, 0.5, 1.0, 1.5, 2.0 mg L^{-1}) of various hormones (2, 4-D, BAP, NAA and IAA). It was observed that nodal explants showed growth response like direct regeneration, enlargement and initiation of callus.

Statistical analysis

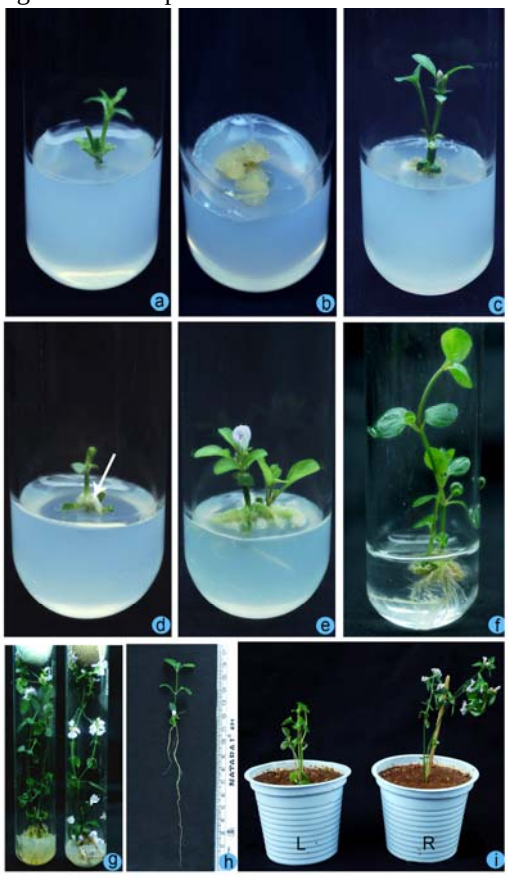
Data were scored after 24 and 45 days for recording multiple shoot induction and rooting frequency, respectively. Data were presented in mean ± S.E. of 10 explants per treatment and repeated three times.

Results and Discussion

Plant regeneration and rooting

Sterilized nodal explant of *J. prostrata* was placed in MS medium supplemented with different concentration of plant growth regulators to induce callus and direct organogenesis (Table-1). The types and concentration of the plant growth regulators in the culture medium influence successful induction of organogenesis (Oggema *et al*., 2007). The use of BAP for initial sprouting of axillary buds from mature nodal explants of *B. edulis*, *B. ventricosa* and *D. asper* have been suggested by several other workers (Lin and Chang, 1998; Huang and Huang, 1995; Arya and Arya, 1996). It was also proved in our present investigation by well developed and highest rate of shoot multiplication was formed in *J. prostrata* through BAP at 0.5 (Fig. 1a) and 1.5 mg L^{-1}. Maity and Ghosh (1997) reported that BAP contributes to organogenesis at lower concentrations, since higher concentrations have negative effect on shoot regeneration and are deleterious to *D. strictus.* Also, Lin and Chang (1998) have demonstrated that higher concentrations of cytokinins promote shoot multiplication in *B. edulis*, but elongation is inhibited and considerable vitrification occurs. The highest shoot length was achieved from lower concentration of BAP (1.5 mg L^{-1}) within 24 days duration. But, the 2 mg L^{-1} of BAP influenced only 2.8 cm of shoot induction. Bag *et al*., (2000) have also reported that increased concentration of BAP adversely affects the rate of shoot multiplication in *T. spathiflorus*. Our present investigation also confirmed that the medium containing 2.0 mg L^{-1} BAP was affected considerable shoot multiplication.

The grown hormone, BAP is said to be the inducer of multiple shooting as described by earlier reports (Jagadesh Chandara *et al*., 1999; Britto *et al*.,2001; Patil,1998),When the regenerated shoots were elongated up to around 2 cm, shoots were excised and transferred in to MS medium fortified with the same medium. Among various hormones, BAP (1 mg L^{-1}) and IAA (0.5 mg L^{-1}) were induced considerable percentage (70%) of direct shoot regeneration of *J.Prostrata.* The medium supplemented with 2, 4-D (Fig.1b) and NAA at different concentrations induced callus formation at nodal region of the explants.



a. Shoot initiation on MS medium with 0.5 mg L^{-1} of BAP, b. Initiation of callus from nodal explants on 0.5 mg L^{-1} of 2, 4- D, c. Callus at proximal end of shoot on 2, 4- D (0.5 mg L^{-1}), d. Root induction on 1.5 mg L^{-1} of NAA, e. Regenerated plantlet with *in vitro* flower on NAA (0.5 mg L^{-1}), f. Plantlet on liquid culture of BAP (0.5 mg L^{-1}), g. *In vitro* flowering on BAP of 0.5 mg L^{-1}, h. Regenerated plantlet with developed root on IAA (0.5 mg L^{-1}), i. Plastic cups contain 24 (L) and 124 (R) days old plantlet.

Fig.1: *In vitro* direct regeneration of nodal explant of *Justicia prostrata*

The different plant growth regulators of BAP, NAA and IAA induced the rooting of *J. Prostrata* shoots (Table 2). MS medium with 2, 4-D and BAP at 0.5 mg L^{-1} concentration induced callus formation at the proximal end of the stem (Fig. 1c). Among auxin and cytokinin, 0.5 mg L^{-1} of NAA was successfully induced the

maximum length of root than BAP. It was previously reported that auxins play an essential role for root induction in shoots of *S.fruticosa* (Arikat *et al*., 2004). As for rooting percentage, NAA was the least successful auxin in our experiments, which is in agreement with the results reported by Arikat *et al*., (2004) for *S.fruticosa* and for *S.valentine* (Cuenca and Amo-Marco, 2000). The MS medium with NAA at 1.5 mg L^{-1} induced root (Fig. 1d) and also *in vitro* flowering was attained on 0.5 mg L^{-1} within 24 days duration (Fig. 1e). Besides, Sanches-Gras and calvo, (1996) reported NAA inhibitory effect on rooting of *Lavandula latifolia*. The highest rooting length was obtained on media containing 0.5 mg L^{-1} of IAA at 24 days old plant (Fig. 1h) and lowest, 2 mg L^{-1} NAA (65%). The *in vitro* flowering was successfully obtained on 0.5 mg L^{-1} of both BAP and NAA (Fig. 1g).

Table -1: Effect of plant growth regulators on callus induction and shoot multiplication from nodal explants of *J. prostrata*. *Data were recorded after 24 days of inoculation.*

Plant growth regulators	Hormone concentration (mg L^{-1})	Percentage of responses (%)	Callus formation	No. of shoots/ Explants	Shoot length (cm)
2-4-D	0.2	10	+	1	0.76±0.03
	0.5	50	+	2	1.7±0.06
	1.0	20	+	2	2.40±0.05
	1.5	70	+	3	2.63±0.06
	2.0	50	+	2	1.83±0.06
BAP	0.2	30	-	2	1.23±0.03
	0.5	90	-	2	2.13 ±0.03
	1.0	70	-	2	4.23±0.03
	1.5	80	-	3	5.43±0.03
	2.0	60	-	2	2.76±0.03
NAA	0.5	60	+	2	4.90±0.05
	2.0	40	+	2	2.56±0.03
IAA	0.5	70	-	3	2.43±0.03
	2.0	50	-	2	1.56±0.03

All values are Mean ± S.E of three experiments. Data represents an average of ten explants per treatment.
+ = Presence, - = Absence

Acclimatization and transfer to soil

Plantlets, with a well-developed root system on 0.5 mg L^{-1} of BAP, were washed carefully to remove agar and then transferred to the pots containing garden soil and sterile vermiculite (1:1 ratio; Fig. 1i). After watering, plantlets were maintained in a growth chamber at 27±1^{o}C under 16 h illuminations with lambs. After 3 weeks of acclimatization had been completed, plantlets were transferred to large pots for further growth.

Results of the present investigation showed that it is possible to regenerate large number of multiple shoots from the nodal explants of *J. prostrata* on MS medium with 0.5 and 1.5mg L^{-1} of BAP. And it also proved that this plant can be grown in single hormone up to *in vitro* flowering and rooting. This protocol would provide an effective strategy for the mass production of *J. prostrata* Gamble.

Table-2*:* Effect of plant growth regulators on root induction of *J. prostrata*. Data were recorded after 45 days.

Plant growth regulators (mg L^{-1})	Culture establishment (%)	No. of roots/ explants	Root length (cm)
BAP			
0.5	90	11	6.73±0.03
1.0	70	8	5.76±0.18
NAA			
0.5	75	9	4.60±0.05
1.0	65	4	3.40±0.05
IAA			
0.5	95	2	11.70±0.05
1.0	80	2	8.21±0.17

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References

Arikat, N.A., Jawad, F.M., Karam, N.S., and Shipli, R.A. 2004. Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill.). *Scientia Hort*., 100: 193 -202.

Arya, I.D. and Arya, S. 1996. Introduction, mass multiplication and establishment of edible bamboo *Dendrocalamus asper* in India. *Indian journal of plant genetic resources*, 9: 115 -121.

Bag, N., Chandra, S., Palni, L.M.S., and Nandi, S.K. 2000. Micropropagation of Dev-ringal (*Thamnocalamus spathiflorus* (Trin.) Munro) - a temperate bamboo and comparison between *in vitro* propagated plants and seedlings. *Plant science*, 156: 125 -135.

Britto, J.S., Robinson. J.J., Natarajan, E., and Arockiasamy, D.I., 2001. Micropropagation of *Hyptis suaveolens* (L.) poir. (Labiatae) through nodal culture. *Ad. Plant. Sci*., 14 (11):561 -565.

Cuenca, S. and Amo-Marco, J.B. 2000. *In vitro* propagation of two Spanish endemic species of *Salvia* through bud proliferation. *In vitro Cell Dev Biol-Plant*., 36:225 -229.

Ghosal, S., Banerjee, S. and Frahm, A.W. 1979. Prostalidins A, B, C and retrochinensin: A new antidepressant: 4-aryl-2, 3 naphthalide lignans from *Justicia prostrata*. *Chem. Ind*., 854 -855.

Huang, L.C. and Huang, B.L. 1995. Loss of the species distinguishing trait among regenerated *Bambusa ventricosa* McClure plants. *Plant cell, tissue and organ culture*, 42: 109 -111.

Jagadesh Chandra, K.S., Rachappji, S., Gowda, K.R.D., and Tharasaraawathi, K.J., 1999. *In vitro* Propagation of *Pisonia alba* (L.) Sanogel. A threatened species. *Phytomorphology*, 49 (1): 43 - 47.

Lin, C.S. and Chang, W.C. 1998. Micropropagation of *Bambusa edulis* through nodal explants of field-grown culms and flowering of regenerated plantlets. *Plant cell reports*, 17: 617- 620.

Maity, S. and Ghosh, A. 1997. Efficient plant regeneration from seeds and nodal segments of *Dendrocalamus strictus* using *in vitro* technique. *Indian forestry*, 4: 313-318.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiology of the plant*, 15: 473-497.

Oggema, J.N., Kinyua, M.G. and Ouma, J.P. 2007. Optimum 2, 4-D concentration suitable for embryogeneic callus induction in Local Kenyan sweet potato cultivars. *Asian J. Plant Sci*., 6: 484 - 489.

Patil, V.M., 1998. Micropropagation studies in *Ceropegia* spp. *In vitro cell. Dev. Biol. Plant*., 34: 240 - 243.

Sánches-Gras, M.C. and Calvo, M.C. 1996. Micropropagation of *Lavandula latifolia* through nodal bud culture of mature plants. *Plant Cell Tissue Organ Culture*. 45: 259 - 261.

Sanmugapriya, E.2001. Chemical and pharmacological evaluation of *Justicia prostrata*, M. Pharm Thesis, Banaras Hindu University, Varanasi, India.

Sanmugapriya, E., Shanmugasundaram, P. and Venkataraman, S. 2003. Antinociceptive effect of aqueous and alcoholic extracts of *Justicia prostrata*. *Biomedicine*, 23: 113 - 117.

Sanmugapriya, E., Shanmugasundaram, P. and Venkataraman, S. 2005. Antiulcerogenic effect of *Justicia prostrata* Gamble. *Ind. J. Exp. Biol.*, 43: 181 - 186.

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Original Article

Genetic analysis of somoclonal variation among *Jasminum auriculatum* (Vohl.) and it's callus

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Abstract

In this research article reveals that gentle stability in tissue cultured *Jasminum auriculatum* was examined by random amplified polymorphic DNA (RAPD) analysis. Calli were obtained from *in vitro* grown *Jasminum* leaf segments on MS medium contains 2, 4-D, kinetin and BAP. Calli were induced from explant on MS medium supplemented with 2.5 mg/l of 2,4-D and 0.90 mg/l of KIN,60 to 70 days after calli induction DNA samples from the 10 randomly selected callus and leaf segments of mother plant were subjected to RAPD analysis the detection putative somoclones . A total of 10 arbitrary sequence primers were evaluated, 5 primer produced a high percentage polymorphic amplification products were observed. Mother explant and 2, 4-D induced calli and indicating a high level of genetic variation. Mother explants of *J. auriculatum* showed the smallest somoclonal variation and it compared to the 2, 4-D induced calli having high level of variation this report demonstrates the feasibility of easy induction of regenerative calli by using combination of 2, 4-D and kinetin and the possibility of detecting genetic variation through RAPD analysis among mother and its regenerative calli of *J. auriculatum*.

Keywords: *Jasminum auriculatum*, somoclonal variation, RAPD-PCR.

ABBREVATION: 2,4-D – dicloro phenoxyacetic acid, BAP- benzyl aminpurine , MS – Murashige and Skoog, CTAB – cetyl trimethylammonium bromide.

Introduction

Identification of possible Somaclonal variants at an early stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants. Somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture conditions (Larkin *et al*., 1981, Muller, *et al*., 1990).

Any genetic change induced by *in vitro* conditions of tissue culture is expected to generate stable plants carrying interesting heritable traits. However, such random changes are not desirable in plant transformation experiments. Therefore, their early detection is considered to be very useful in plant tissue culture and transformation studies. Randomly amplified polymorphic DNA (RAPD) based detection of genetic polymorphism (Welsh,*et al*.,1990, Williams,*et al*.,1990) has found successful application in describing somaclonal variability in regenerated individuals of several plant species (Isshiki, *et al*., 1993, Munthali, *et al.,*1996,Hashmi, *et al* 1997).

Jasminum auriculatum is distributed in the western peninsula of India. It is native of south India and the central provinces. A large number of *j. auriculatum* species are centered on the regions comprising India, China, and Malaysia *(*Khoder *et al.,* 1979)*. J. auriculatum* belongs to the family oleaceae. Climbing shrubs 2 to 3 m in height; branches pubescent. Leaves, shiny auricles, trifoliate; terminal leaf let up to 3.5×1.3 cm, ovate, subglarous, obtusely acute and macronate at apex, rounded at base. The lower leaf let small and frequently. Flowers are white, sweet scented borne in pubescent ,compound many flowered flax cymes ,trichotomous or paniculate cymes ,globose. Corolla lobes are elliptical and fruits black. *(*Muthuswami *et al*., 1972).

Flowers are used for production of perfumes. There is a great demand for jasmine

absolute and concrete in perfume, soap and cosmetic industry, oilmen's and are sold in the market at exorbitant price (Mohamad alikhan *et al.*,1989).

In the present paper, we report successful induction of regenerative calli from *J.auriculatum* leaf explants, cultured on Murashige and Skoog's (MS) medium supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid; as an auxin) and benzylademino purine (BAP; as cytokines), kinetin and the extent of genetic variability in the plants regenerated from one of these calli as examined through RAPD analyses.

Materials and Methods

Jasminum auriculatum Vahl procured from St Xavier's College, Palayamkottai were used in this study. Leaf explants, from a field-grown plant (mother plant), were sequentially washed under running water and with Tween - 20 for 10 min each. Their surface was disinfected with by treated with 0.1% w/v mercuric chloride solution for 3 min. Finally, they were washed 3–4 times with sterile distilled water and inoculated aseptically on MS basal medium (Murashige, T. and Skoog, 1962) containing combinations of 2, 4-D (0.5 to 2.5mg/l) and kinetin (0.1 to 0.9 mg/l). Regeneration of calli was attempted on MS medium containing 2, 4-D (2.5 mg/l) and kinetin (0.9 mg/l). The pH of all media was adjusted to 5.8 and 0.8% weight/volume) agar was added prior to autoclaving at 103 kpa for 20 min.Cultures were incubated under a 12 h photo-period with light intensity of 3000lux at 26 ±1°C.

PCR analysis

For DNA extraction, approximately 1 g fully grown calli was grounded using a hand held grinder with liquid nitrogen and then extracted using cetyl trimethyl ammonium bromide (CTAB) buffer(Doyle and Doyle 1990). The amount of DNA and its quality were assessed by UV spectrophotometer. The DNA is pure enough (OD260/280 =1.68), (Sambrook and Russel, 2000) for RAPD- PCR analysis (Williams *et al*., 1990).

Earlier, ten primers had been tested and five primers which produced reproducible bands and they were selected. The experiments were repeated three times and confirmed the reproducibility of bands. PCR reactions were carried out in a total volume of 20µl at a final concentration of 1 mM $MgCl_2$, 2mM dNTP, *Taq* DNA polymerase enzyme (1u/20µ), with approximately 200 ng DNA as a template and a single random primer (0.2 mM). Conditions were 94°C for 2 min, one cycle, 94°C for 15 sec, 35°C for 15 sec, 72°C for 30sec which were repeated in 40 cycles followed by 5 min-extension at 72°C. Then 8µl of each PCR product was revealed on 1% agarose gel subjected to electrophoresis at 80V after staining using ethidium bromide by UV transilluminator and photographed with gel documentation system Alpha Imager 1200.

Results and Discussion

Previously, a variety of experiments were conducted to select plant growth regulators to establish medium requirements for callus culture in ornamental *Jasminum auriculatum* tissue culture. 2,4-D was effective for inducing callus proliferation in this species. It seemed of interest to screen for the presence of Somaclonal variation in regenerates were obtained in medium supplemented with different 2,4-D concentrations.

High-quality DNA was isolated from the ornamental *J. auriculatum* leaves. Evaluation of Somaclonal variation by RAPD-PCR showed that at least 5 out of 10 primers could reveal some polymorphism in the amplified DNA pattern caused by 2,4-D. The patterns of DNA amplification using different primers are shown in Plate 1-D.

Table -1: Shows the primers, primer sequences, number of bands produced and total number of amplicons revealed by RAPD analysis of its callus.

Primers	Sequences 5'-3'	Total no. of bands	No. of polymorphic bands
OPB05	TGCGCCCTTC	5	-
OPB14	TCCGCTCTGG	6	-
OPB20	GGACCCTTAC	6	1
OPB16	TTTGCCCGGA	5	-
OPB17	AGGGAACGAG	7	1

Table-2: Summary of random amplified polymorphic DNA (RAPD) products from callus and original plant of *J. auriculatum* used in the present study.

Description	RAPD
Number of primers tested	10
Number of primers selected	5
Number of primers that showed polymorphisms	2
Total number of amplified bands	60
Total number of monomorphic amplicons	58
Total number of polymorphic amplicons	2
Percent of polymorphic bands	1.2%
Size of amplified bands	
Average number of polymorphic bands per primer	0.4
Average number of bands per primer	12

Table-3: Presence (+) or absence (-) of polymorphic RAPD bands generated by primers in *J.auriculatum* cultured in media with different 2,4-D concentrations, after 120 days.

S.No.	Polymorphic regenerants	
Primer	Mother plant	Callus
OPB 05	1	-
OPB14	0	-
OPB20	1	1
OPB16	1	0
OPB17	0	1

The 5 primers produced 60 well-defined fragments; among them, 58 bands were monomorphic (98.8%) and 2 bands were polymorphic (1.2%) (Table 2). Genetic variability in propagules of ornamental *Jasminum* of 0.4 bands per primer. The length of the amplification products was between 300 and 1000 bp. The number of bands per primer varied from 5 (OPB-05) to 7 (OPB-17) (Table 1). The polymorphic fragments were produced by 2 primers (OPB-20 and OPB-17). Among the primers that identified polymorphisms, each primers produced one polymorphic band (Table-1). The polymorphism generated by OPB-20 and OBP-17 were observed in single samples with genetic variation (9%). OPB-20 and OBP-17 independently identified one Somaclonal variant (Table-3). Examples of RAPD profile of mother plant and callus are shown in Figure 1-C.

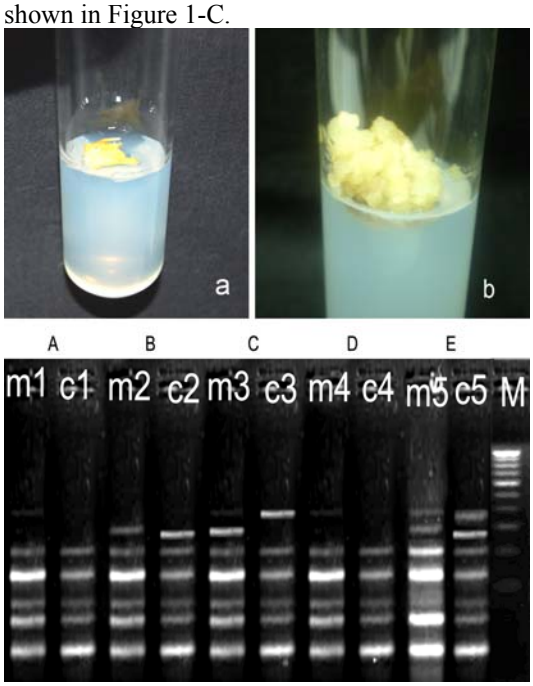

Plate: 1a-c: Molecular pattern of callus revealed by the RAPD with the primers A -OBP-5; B -OBP-14; C- OBP20; D- OPB-16; E -OBP-17; M-markers : m1,m2,m3,m4 and m5 indicates mother plants; c1, c2,c3, c4, and c5 indicate callus

The percentage of polymorphism detected *J. auriculatum* in 2,4-D containing medium was estimated to be 1 to 2 % confirming the occurrence of variation during the callus induction process. The variation of the electrophoretic pattern found in genomic DNA of callus, after 120 days of culture, was probably due to the medium supplementation with 2,4-D (Costa and Zaffari (2005). In *Ananas bracteatus* cv. *striatus*, phenotypic variations of 52% albino plants and 20.5% green plants were described by Costa and Zaffari (2005). In commercial pineapple, Feuser *et al*. (2003) observed a more reduced rate of somaclonal variation among plantlets regenerated from *in vitro* culture using either stationary liquid medium or a temporal immersion system. These authors utilized 10 RAPD primers and detected 7.5 and 5.0% of somaclonal variants for the stationary and temporal immersion systems, respectively. However, in this latter study, the authors did not state the period of culture in which the

samples were taken for RAPD analysis. The culture period directly influences the appearance of somaclonal variation (Skirvin *et al.,* 1994), and the range of variation of 1 to 3% is expected in the process of micropropagation. Some authors have indicated that the treatment used in tissue culture, with high growth rate, may increase the variant numbers (Bairu *et al.,* 2006). The regeneration systems from organized meristems, such as shoot tip and axillary buds, are considered to be the most efficient methods to guarantee genetic integrity of the micropropagated material. The regeneration methods from leave explants (Kawiak and Lojkowska, 2004) and callus (Skirvin *et al.,* 1994) are considered to be less stable permitting the occurrence of genetic variation. In *Drosera binata*, plantlets regenerated through shoot tip preserve the genetic integrity of micropropagated plants. In *Curcuma longa*, rhizome bud explants used to establish cultures show genetic homogeneity in the regenerated propagules, when comparing them with the mother plant (Tyagi *et al*., 2007). However, plants regenerated from leaf base callus have shown variation at the DNA level during *in vitro* culture (Salvi *et al.,* 2001; Tyagi *et al*., 2007).

In this study, RAPD was effective in showing the variations that may occur as a result of mutations during the callus culture of *J. auriculatum* in 2,4-D supplemented medium, and that could be useful in detecting the presence of genetic variation in the initial stages of callus development. In conclusion, the present investigation indicated that mass propagation via tissue cultures produces clones genetically similar to the mother plants. It also conclude that RAPD approach is convenient, fast and reproducible to detect the presence of genetic variation associated with tissue culture of *J .auriculatum* also, these findings could be applied in the breeding of this species.

References

Bairu, M.W., Fennell, C.W. and Van Staden, J. (2006). The effect of plant growth regulators on somaclonal variation in Cavendish banana (*Mus*a AAA cv. '*Zelig*'). *Sci. Hortic.*109: 347-351.

Costa, F. and Zaffari, G.T. (2005). Micropropagação de *Ananas bracteatus* (Shultz) cv. *striatus* Hort. *Rev. Bras. Hort. Ornamental.* 11: 109-113.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus.* 12:13-15.

Feuser, S., Meler, K., Daquinta, M. and Guerra, M.P. (2003). Genotypic fidelity of micropropagated pineapple (*Ananas comosus*) plantlets assessed by isozyme and RAPD markers. *Plant Cell Tissue Organ Cult.* 72:221-227.

Isshiki, S., Okubo, H. and Fuziada, K. (1994). Phylogeny of eggplant and related *Solanum* species constructed by allozyme variation. *Sci. Hort*. 59:171–176.

Karihaloo, J. L. and Gottlieb, L. D. (1995). Random amplified polymorphic variation in the eggplant, *Solanum melongena* L. (Solanaceae).*Theor. Appl. Genet*. 90:767–770.

Kawiak, A. and Lojkowska, E. (2004). Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* plantlets. *In Vitro Cell. Dev. Biol. Plant.* 40: 592-595.

Larkin, P.J. and Scowcroft, W.R. (1981). Somaclonal variation: a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet*. 60: 197-214.

Mohamad Alikhan,W., Raman, V.S and Raman K.R (1989). New chromosomal forms of superior ornamental values in Jasmines. *South Indian hort*. 17:79-83.

Muller, E., Brown, P.T.H., Hartke, S. and Lorz, H.T.I. (1990). DNA variation in tissue-culture-derived rice plants. *Theor Appl Genet*. 80: 673–679.

Munthali, M., Ford-lloyd, B.V. and Newbury, H.J. (1992). The random amplification of polymorphic DNA for fingerprinting of plants. *PCR Methods Appl*. 1: 274-276.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.

Muthuswami, S., Thangaraj, T. and Pappiah, C.M. (1972). Variation in flower characters of J.auriculatum Vahl. seedling . *South Indian Hort.* 20:87-92.

Ramdas, S., Peter, G.B. and Muthuswami, S. (1993) Jasmine In: Bose TK.& Yadav LP (eds) *Commercial Flowers*. Naya Prokash, Calcutta. 486–517.

Salvi, N.D., George, L. and Eapen, S. (2001). Plant regeneration from leaf base callus of

turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell Tissue Organ Cult.* 66:113-119.

Sambroog, J . and Russel (2000). Molecular cloning : *A laboratory manual third edition* .Cold Spring Harbour Laboratory press.Newyork:78-85.

Skirvin, R.M., McPheeters, K.D. and Norton, M. (1994). Sources and frequency of somaclonal variation. *HortScience* 29:1232-1237.

Tyagi, R.K., Agrawal, A., Mahalakshmi, C. and Hussain, Z. (2007). Low-cost media for *in vitro* conservation of turmeric (*Curcuma longa* L.) and genetic stability assessment using RAPD markers. *In Vitro Cell Dev. Biol. Plant.* 43:51-58.

Welsh, M. and McCleland, M. (1991). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acid Res*. 18:7213-7218.

Williams, J. G. K A. R., Kubelik, K. J., Livak, J. A., Rafalski and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. 18:6531– 6535.

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Original Article

Wound healing effect of chitosan in fresh water fish *Cyprinus carpio L.*

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Abstract

The shell fish (crustacean) processing industry in India generates 8.5 million tones of waste per year. These wastes have an appreciable potential for pollution and do pose a disposal problem. These wastes were converted in to chitin (value added bio polymer) and chitosan by demineralizing, deproteinizing and deacetylating with 40 - 52% NaOH for 2hr boiling at 110°C. Chitin / chitosan were renounced for its commercial application in biomedical, food preservation and chemical industries. In this study chitin / chitosan was used as a dietary supplement with basal diet and tested in *Cyprinus carpio* L. Effect of chitin / chitosan on wound healing in fingerling and adult *C. carpio* were studied. α-chitosan feeding promotes epithelialization, faster wound contraction and reformation of connective tissue and angiogenesis. The present study revealed that the use of α- chitosan as wound healer of carps in intensive culture conditions.

Keywords: Crustacean; chitin; chitosan; *Cyprinus carpio*

Introduction

Wound healing is the process of repair that follows injury to the skin and other soft tissues. The capacity of a wound to heal depends in part on its depth, as well as on the overall health and nutritional status of the individual (Enoch and Harding, 2003). Dietary modifications, with and herbal supplements may improve the quality of wound healing by influencing the regenerative processes or by limiting the damaging effects of inflammation (Davis *et al*., 1989).

Glucosamine sulfate and chondroitin sulfate may both play a role in wound healing by providing the raw material needed by the body to manufacture connective tissue found in skin, tendons, ligaments and joints (Morrison and Murata,1974). One controlled trial in human found that wounds healed with greater strength when they were treated topically with a chondroitin sulfate-containing powder (Prudden *et al.,* 1969). Arginine supplementation increases protein synthesis and improves wound healing in animals (Barbul *et al*., 1983). Two controlled trials have shown increased tissue synthesis in surgical wounds in people given 17 - 25 grams of oral arginine per day (Krik *et al*., 1993; Barbul *et al*., 1990).

Chitosan was used in wound dressing matrices (Jayasree *et al.,*1995) and was shown to be a wound healing accelerator (Minami *et al.,* 1993). Chitosan's cationic nature appeared to be the main mechanism by which cells are attracted to this polymer; however, the degree of cell attachment is attributed to the percent deacetylation of the chitosan. Prasitslip *et al.* (2000) studied how degree of deacetylation affected *in vitro* cellular responses of chitosan from two different sources, shrimp and cuttle fish. They tested four chitosan substrates, two from each source, differing by about 10% in deacetylation and ranging between 76% and 90% deacetylation. Results indicated that cells more readily attached to more deacetylated chitosans from both sources. The *in vivo* chitosan tissue biocompatibility studies tended to be in agreement with the *in vitro* cell response studies. *In vivo* studies of tissue response to films of chitosan showed a marked increase in chitosan-tissue biocompatibility as chitosan deacetylation increased (Tomihata and Ikada, 1997). The present study examines the effect of chitin and chitosan supplemented diets on the proliferation of fish epithelial cells and time in healing of wounds in *Cyprinus carpio* (L.).

Material and Methods

Experimental design

Cyprinus carpio fingerlings of mean body weight of 56.9 ± 0.5 g were randomly distributed into six 250 liter tanks supplied with partially recirculating ground water at 25°C. The experimental tanks were assigned to each of four dietary treatments containing 1%each of α, β-

chitin, *α, β*- chitosan. After 6 weeks of acclimation *C. carpio* were fed with basal diet and basal diet supplemented with 1%each of α, β- chitin and *α, β*- chitosan. The fish were fed with the experimental diets for a period of 4 weeks. At the end of the experimental period, fish were experimentally wounded and the responses of wound closure were observed for another 3 weeks by feeding the basal and chitin/ chitosan supplemented diets. Wound infliction consisted of a small incision measuring 1.5 ± 0.2cm in length and 0.3cm in depth made on each lateral side of the fish with a scalpel and a plastic guide. The incision penetrated the epidermis, dermis and underlying musculature. Measurement of wound closure was as per earlier descriptions (Lim *et al.,* 2004).

$$\textit{Wound closure} = \frac{\text{Final wound (cm)}}{\text{Initial wound (cm)}} \times 100$$

Wound from individual fish was photographed every day, beginning on the day of wounding. Wound size was then calculated by determining the area of the wound in comparison to the standard. Wound closure was expressed as the ratio of wound area (each day after wounding). A smaller wound ratio indicated faster wound closure.

Statistical analysis

Data were reported as mean ± standard deviation. Statistical significance of the influence of diet on wound size were determined at each time using Student '*t*' test at 5% level of significance.

Wound healing influence of chitin/ chitosan supplemented diet was studied in *C. carpio* fingerlings for a period of 35 days. The experimental fishes fed with *α*- chitosan supplemented diet showed significant reduction in the wound dimensions than all other experimental diets. On 35th day the wound was completely healed in α- chitosan supplement diet only. About 3.70% was cured in the fishes fed with basal diet after 35th day. 31.25% was healed in the fishes fed with α- chitin supplement diet and 26.67% wound closure was observed in fishes fed with *β*- chitin supplemented diet. After 35th day, 68.75% wound closure was observed in β- chitosan supplemented carps. Significant wound healing was observed in *α, β*- chitosan, *α*- chitin supplemented diets fed *C. carpio* fingerlings after 35 days (P<0.05) (Table - 1).

Wound healing effect in adults carps

Wound healing influence of chitin/ chitosan supplemented diet was studied in adult carps for a period of 28 days. The experimental fishes fed with *α*- chitosan supplemented diet showed significant reduction in the wound dimensions than all other experimental diets. On 28th day the wound was completely healed in α- chitosan supplement diet, only about 6.670% was cured in the fishes fed with basal diet after 28th day, 43.75% was healed in the fishes fed with α- chitin supplemented diet and 40.00% wound closure was observed in fishes fed with *β*- chitin supplemented diet. After 28th day significant response in (93.33%) wound closure was observed in *β*- chitosan supplemented carps. Wound healing was significant in *α, β*- chitosan, *α*- chitin supplemented diets fed *C. carpio* after 28th days (P<0.05) (Table - 2).

Result

Wound healing responses in fingerlings

Table - 1: *C. carpio*: Wound closure responses of fingerlings fed with chitin / chitosan supplemented diet for a period of 35 days

Feed type	Wound size (cm)						Healing response
	Initial	Day 7	Day 14	Day 21	Day 28	Day 35	(%)
Basal diet (BD)	1.35	1.35	1.40	1.50	1.40	1.3	3.70
BD + α- chitin	1.60	1.55	1.40	1.30	1.20	1.10	31.25*
BD + β- chitin	1.50	1.45	1.40	1.30	1.20	1.10	26.67*
BD + α- chitosan	1.60	1.40	0.95	0.61	0.28	C. H	C. H *
BD + β- chitosan	1.60	1.45	1.20	0.95	0.75	0.50	68.75*

C. H - Completely healed, * indicates significance (P< 0.05) level.

Table - 2: *C. carpio*: Wound closure responses of adult fish fed with chitin / chitosan supplemented diet for a period of 28 days

Feed type	Wound size (cm)					Healing Response (%)
	Initial	Day 7	Day 14	Day 21	Day 28	
Basal diet (BD)	1.50	1.55	1.50	1.45	1.40	6.67
BD + α- chitin	1.60	1.50	1.30	1.10	0.90	43.75*
BD + β- chitin	1.50	1.40	1.30	1.10	0.90	40.00*
BD + α- chitosan	1.60	1.10	0.60	0.25	C. H	C. H *
BD + β- chitosan	1.50	1.10	0.85	0.45	0.10	93.33*

C. H – Completely healed, * indicates significance (P< 0.05) level.

Discussion

Healing of skin wound was quite a complicated process involving epidermal regeneration, fibroblast proliferation, neovascularization and synthesis. Many investigators studied the acceleration of wound healing process and shortening of healing period. Supplementation of chitin/ chitosan diet improved the wound healing efficiency of *C. carpio* and accelerated healing period (Conti *et al*., 2000). Chitosan helped the regeneration of tissue elements in skin wound and had positive effects on wound healing (Bartone and Adickes, 1988).

Supplementation of α and β- chitosan in the diet significantly improved the wound closure in the experimental carps. Similarly application of water soluble chitosan solution resulted in complete re-epithelialization, fibrosis and regrowth of hair follicles within 7 days after wounding (Cho *et al*., 1999). Ueno *et al*. (1999) reported that on the 3rd day after wounding, the chitosan treated wounds showed heavy infiltration of polymorphonuclear cells and an increase in the diffusion compared with other experimental groups in dogs. Granulation was more intense after chitosan treated dog on day 9th and 15th day of post wounding (Ueno *et al*., 1999).

It was anticipated in the experimental studies whether the duration of wound healing could be shortened. Some studies had revealed that exogenous application of growth factors decreased the healing period (Fu *et al*., 1998; Gilpin *et al*., 1994). Quilhac and Sire (1999) examined the dynamics of re-epithelialization process after wounding of cichlids fish and observed a rapid differentiation of the epidermal basal layer cells. In comparison with the epidermis, repair of dermal and muscle structure took much longer and they not reached steady state level in wound site within the period investigated. This finding was supported by Halver (1972) who found a prolonged repair period for epidermal repair. The 15th day after operation untreated control group did not heal and covered with large crust in wounded dog (Kweon *et al*.,2003). Application of water soluble chitosan ointment at wound site may not completely resulted in the healing of wound but improved the healing process and decreased the size of lesion than that of control group (Kweon *et al*., 2003).

Wound healing experiments using this fish model (*C.carpio*) showed that the application of dietary supplementation of chitin/ chitosan on to an open wound induces significant wound contraction and accelerates the wound closure and healing time. The present study revealed that the use of α- chitosan as wound healer of carps in intensive culture conditions.

References

Barbul, A., Lazarou, S.A. and Efron, D.T.,1990. Arginine enhances wound healing and lymphocyte immune responses in humans. *Surgery*, 108: 331-337.

Barbul, A., Rettura, G., and Levenson, S.M.,1983. Wound healing and thymotropic effects of arginine: a pituitary mechanism of action. *Am. J. Clin. Nut.*, 37: 786-794.

Bartone, F.F. and Adickes, E.,1988. Chitosan: effects on wound healing in urogenital tissue: Preliminary Report. *J. Urol*., 140: 1134-1137.

Cho, Y.W., Cho, Y.N., Chung, S.H., Yoo, G. and Ko, S.W.,1999. Water-soluble chitin as a wound healing accelerator. *Biomaterials*, 20: 2139-2145.

Conti, B., Giunchedi, P., Genta, I. and Conte, U.,2000. The preparation and *in vivo* evaluation of the wound-healing properties of chitosan microspheres. *STP. Pharm. Sci*., 10: 101-104.

Davis, R.H., Leitner, M.G., Russo, J.M. and Byrne, M.E.,1989. Wound healing. Oral and topical activity of *Aloe vera*. *J. Am. Podiatr. Med. Assoc*., 79: 559-562.

Enoch, S. and Harding, K.,2003. Wound bed preparation: The science behind the removal of barriers to healing. *Wounds*, 15(7): 213-229.

Fu, X., Shen, Z., Chen, Y., Xie, J., Guo, Z. and Zhang, M.,1998. Randomised place controlled trial of use of topical recombinant bovine basic fibroblast growth factor for second-degree burns. *Lancet,* 352: 1661-1664.

Gilpin, D.A., Barrow, R.E., Rutan, R.L., Broemelling, L. and Herndon, D.N.,1994. Recombinant human growth factor hormone accelerates wound healing in children with large cutaneous burns. *Ann. Surg.*, 220: 19-24.

Halver, J.E.,1972. The role of ascorbic acid in fish disease and tissue repair. *Bull. Jpn. Soc. Sci. Fish.,* 38: 79-92.

Jayasree, R.S., Rathinam, K. and Sharma, C.P.,1995. Development of artificial skin (Template) and influence of different types of sterilization procedures on wound healing pattern in rabbits and guinea pigs. *J. Biomater. Appl.,* 10: 144-162.

Krik, S.J., Hurson, M. and Regan, M.C.,1993. Arginine stimulates wound healing and immune function elderly human beings. *Surgery*, 114: 155-160.

Kweon, D.K., Song. S. and Park, Y.Y.,2003. Preparation of water soluble chitosan/heparin complex and its applications as wound healing accelerator. *Biomaterials*, 24(9): 1595-1601.

Lim, Y., Levy, M. and Bray, T.M.,2004. Dietary zinc alters early inflammatory responses during cutaneous wound healing in weanling CD-1 mice. *J. Nutr.,* 134: 811-816.

Minami, S., Okamoto, Y. and Tanioka, S.,1993. Effects of chitosan on wound healing. C*arbohydrates and Carbohydrate polymer*. Yalpani, Chicago, IL: ATL Press, pp.76-83.

Morrison, L.M. and Murata, K.,1974. Absorbtion, disturbtion, metabolism and excretion of acid mucopolysaccharides administered to animals and patients. In: Morrison, L.M, Schkeide, O.A., Meyar, K. *Coronary heart disease and the mucopolysaccharides*. Charles, C. Thomas, Springfield, pp.109-127.

Prasitslip, M., Jenwithisuk, R., Kongsuwan, K., Damrongchai, N. and Watts, P., 2000. Cellular responses to chitosan *in vitro*: the importance of deacetylation. *J. Mat. Sci. and Mat. Medi.,* 11: 773- 78.

Prudden, J.F., Wolarsky, E.R. and Balassa, L.,1969. The acceleration of healing. *Surg. Gynecol. Obset*., 128: 1321-1326.

Quilhac, A. and Sire, J.Y.,1999. Spreading, proliferation and differentiation of the epidermis after wounding a cichlid fish, *Hemichromis bimaculatus*. *Anat. Rec*., 254: 435-451.

Tomihata, K. and Ikada, Y.,1997. *In vitro* and *in vivo* degradation of films of chitin and its deacetylated derivatives. *Biomaterials,* 18(7): 567-575.

Ueno, H., Yamada, H., Tanaka, I., Kaba, N., Matruura, M., Okumura, M., Kadosawa, T. and Fujinaga, T.,1999. Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs. *Biomaterials,* 20: 1407-1414.

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Original Article

In vitro Antibacterial activity of three Indian medicinal plants

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Abstract

The antibacterial activities of petroleum ether, acetone, ethanol and methanol extracts of three medicinal plant species namely, *Blepharis maderasphatensis Dipteracanthus prostrates* and *Allamanda cathartica* were tested against ten bacterial strains using agar diffusion method. The methanol stem extracts of *D. prostratus* showed significant activity (27 mm) against *Pseudomonas aeruginosa.* The acetone, ethanol and methanol extracts of all the three medicinal plants showed less inhibition zone against *E.coli*. The petroleum ether and acetone stem extracts of *B. maderasphatensis* showed high inhibition zone against *Staphyllococcus sp*, respectively. Hence, the selected three medicinal plants can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals for therapeutic needs.

Key words: *Dipteracanthus prostrates*; *in vitro*; agar diffusion method; natural products

Introduction

Herbal medicine is the oldest form of health care known to mankind. Herbs have been used by all cultures throughout the history and they constitute an integral part of the development of modern civilization. In terms of using plant materials for traditional medicine, it is estimated that local communities use over 7,500 species of plants (Anonymous, 1994; Arora, 1997). Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject for very intense pharmacological studies; this has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutics value and as sources of lead compounds in the drug development. In developing countries, it is estimated that about 80% of the population rely on traditional medicine for their primary health care. There arises a need therefore to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies. In recent years, multiple drug/chemical resistance in both human and plant pathogenic microorganisms have been developed due to indiscriminate use of commercial antimicrobial drugs/chemical commonly used in the treatment of infectious diseases. This situation has forced scientists to search new antimicrobial substances in various sources like medicinal plants (Kumar *et al*.,2006). Medicinal and aromatic plants and their essences are rich in antibacterial compounds which could be an alternate way to combat bacterial diseases even against some bacteria which are becoming resistant to certain synthetic medicines (Meera *et al*., 1999; Ahmad *et al*., 1998; Aswal *et al*., 1996).

The present investigation was undertaken to evaluate the antibacterial activity of three Indian medicinal plants such as *Dipteracanthus prostratus, Allamanda cathartica* and *Blepharis maderasphatensis* against selected bacterial pathogens.

Materials and Methods

Preparation of plant extracts

The dried and powdered plant material (100 g) was extracted successively with 600 ml water, chloroform or methanol with a Soxhlet extractor for 48 h at temperature not exceeding the boiling point of the solvent (Lin *et al*., 1999). The extracts were filtered through Whatman No. 1 filter paper and then concentrated in a vacuum at 40°C using a rotary evaporator. Each extract was transferred to glass vials and kept at 4°C before use.

Bacterial Strains

Ten bacterial strains were used namely *Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Serratia marcescens, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus, Entrobacter aerogens,*

Bacillus subtilis and *Staphyllococcus sp*. The bacterial strains were supplied by the Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India.

Antibacterial assay

The disc diffusion assay methods of Iennette (1985) as described by Rosoanaivo and Ratsimanaga- Urverg (1993) and Rabe and Van Staden (1997), with modifications, were used to determine the growth inhibition of bacteria by plant extracts. Diluted bacterial culture (100 µl) was spread over nutrient agar plates with a sterile glass L-rod. 100 µl of the each extracts were applied to each filter paper disc (Whatman No. 1, 6 mm diam.) and allowed to dry before being placed on the agar plate. Each extract was tested in triplicate (3 discs/plate) and the plates were inoculated at 37±1°C for 24 h. After incubation, the diameter of inhibition zones was measured with a caliper.

Results and Discussion

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay (Tona, *et al*., 1998). Table 1 and 2 reveals that antibacterial activity of stem and leaf explants of three medicinal plants such as *D. prostratus*, *A.. cathartica* and *B. maderasphatensis* were evaluated against ten bacterial pathogenic strains. The acetone (7 mm), ethanol (7 mm) and methanol (8 mm) extracts of *Blepharis maderasphatensis*, *Dipteracanthus prostratus* and *Allamanda cathartica* showed less inhibition zones against *E.coli.* The ethanol extracts of *Blepharis stem* (17 mm), *Alamanda* leaf (12 mm) and the methanol extracts of *Dipteracanthus prostratus* stem (15 mm) and acetone extracts *Alamanda* stem (12 mm) showed minimum inhibition zones against *Serratia marcescens.* Among all the solvents the methanol extract of *Blepharis* stem (11 mm) and acetone extract of *Dipteracanthus* stem (9 mm) showed minimum antimicrobial activity.

Table -1: Antibacterial activity of leaf and stem extracts of some medicinal plants (Inhibition zone in diameter including disc (mm)

Bacterial strains	*B. maderasphatensis*								*D. prostratus*							
	Stem				Leaf				Stem				leaf			
	A	M	E	P	A	M	E	P	A	M	E	P	A	M	E	P
E. coli	–	–	7	–	7	8	10	–	8	8	7	–	–	6	–	–
S. marcescens	–	8	17	–	8	–	12	6	8	15	–	–	–	10	9	6
E. aerogens	8	11	–	–	7	7	8	–	9	–	–	–	–	–	–	–
B. subtilis	–	–	7	–	–	7	8	–	–	7	9	–	–	6	–	–
S. typhi	–	6	8	–	6	–	7	6	–	6	7	–	–	6	9	–
Staphyllococcus sp	21	–	6	–	7	6	–	24	6	8	6	–	7	7	–	–
P. aeruginosa	6	6	6	–	–	7	6	–	–	27	6	–	–	7	9	–
P. mirabilis	6	7	–	–	–	–	–	–	–	6	–	9	–	–	–	–
K. pneumoniae	12	6	8	–	7	6	–	–	–	–	8	–	–	–	7	–
S. aureus	8	16	–	–	11	12	16	–	7	–	9	–	–	–	–	–

A-Acetone extract; M- Methanol extract; E- Ethanol extract; P- Petroleum ether extract
Each experiment was repeated thrice

The ethanol and methanol extracts of *Blepharis* leaf and *Dipteracanthus* stem showed same inhibition zone (7-9 mm) against *Bacillus subtilis.* In both plants *viz., B. maderasphalensis* and *D. prostratus* extracts of ethanol and methanol showed the inhibition zone ranging from 6- 8 mm. Most of the extracts of three medicinal plants showed varying inhibition zone ranging from 6- 12 mm against *Staphyllococcus sp*. The acetone (6 mm), methanol (6 mm) and ethanol (6 mm) extracts of *Blepharis maderasphatensis* and ethanol (6 mm) methanol (7 mm) extracts of *Dipteracanthus prostratus* and finally acetone (9 mm), ethanol (8 mm) extracts of *A.cathartica* showed minimum inhibition zone against *Pseudomonas aeruginosa.* The methanol (27 mm) extracts of stem of *Dipteracanthus prostratus* showed highest inhibition zone against *Pseudomonas aeruginosa* than other extracts.

Table -2: Antibacterial activity of leaf and stem extracts of *A. cathartica* (Inhibition zone in diameter including disc (mm)

Bacterial strains	Chloram phenicol (30 mg/Disc)	*A. cathartica*							
		Stem				Leaf			
		A	M	E	P	A	M	E	P
E. coli	24	–	–	–	–	7	–	7	–
S. marcescens	33	12	–	6	–	–	6	12	–
E. aerogens	40	–	–	–	–	–	–	–	–
B. subtilis	25	7	–	6	–	6	7	6	–
S. typhi	22	–	–	–	–	–	–	–	–
Staphyllococcus sp	21	8	7	11	–	–	8	13	–
P. aeruginosa	32	9	–	6	–	6	–	8	–
P. mirabilis	27	–	6	–	–	–	6	–	–
K. pneumoniae	32	–	7	7	–	–	9	11	6
S. aureus	24	–	11	7	–	–	14	–	–

*Each experiment was repeated thrice; A. Acetone extract; M- Methanol extract; E- Ethanol extract; P- Petroleum ether extrac*t

The acetone (6 mm), ethanol (7 mm) stem extracts of *Blepharis maderasphatensis*, petroleum ether (9 mm), methanol (6 mm) stem extracts of *Dipteracanthus prostratus* and only the methanol extracts of both stem and leaf of *A. cathartica* inhibits *Proteus mirabilis* with less inhibition zone (6- 9 mm). Among various extracts, the acetone (12 mm) stem extracts of *Blepharis maderasphatensis* showed significant inhibition zones against *Klebsiella pneumonia* and less inhibition zone was obtained in ethanol (7 mm) and methanol (9 mm) extracts of *Allamanda cathartica* leaf. However, to know the exact mechanism of action of *B. maderasphatensis, D. prostratus* and *A. cathartica* extracts, further studies with purified fractions/bioactive compounds are necessary.

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References

Ahmad I., Mehmood Z., and Mehmood I., 1998. Screening of some Indian medicinal plants for their antimicrobial properties. *J. Ethnopharmacol.*, 62: 183- 193.

Anonymous, 1994. *Ethnobotany in India- A status report*. All India coordinated research project in Ethnobotany, Ministry of Environment and Forests, Govt. of India, New Delhi.

Arora R.K., 1997. Ethnobotany and its role in the conservation and use of plant resources in India. *Ethnobotany*, 9: 6-15.

Aswal B.S, Goel A.K, and Patneik G.K, 1996. Screening of Indian medicinal plants for biological activity. *Indian J. Exp. Biol.*, 34: 444- 467.

Iennette E.H. 1985. *Manual of clinical microbiology*, 978–987. 4th edn. American Association for Microbiology, Washington.

Kumar R. S., Sivakumar T., Sundaram R.S., Sivakumar P., Nethaji R., Gupta M., and Mazumdar U.K., 2006. Antimicrobial and Antioxidant Activities of *Careya arborea* Roxb. Stem Bark. *Iranian journal of pharmacology & therapeutics*, 5:35-41.

Lin J, Opak War, and Geheeb-Keller M. 1999. Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and antimicrobial activities. *Journal of Ethnopharmacology*, 68: 267–274.

Meera P, Dora P.A., and Samuet J.K. 1999. Antibacterial effects of selected medicinal plants on the bacteria isolated from Juices, *Geobios*, 26, 17- 20.

Rabe T., and Van Staden. (1997). Isolation of an antibacterial sesqui-terpenoid from *Warbugia salutaris*. *Journal of Ethnopharmacology* 73: 171–174.

Rosoanaivo and Ratsimanaga- Urverg 1993. Biological evaluation of plants with reference to the Malagasy flora. Monograph for the IFs. NAPRECA Workshop on Bioassays. Antananavivo, Madagascar, 72–79.

Tona, L., Kambu K., Ngimbi N., Cimanga K., and Vlietinck A.J., 1998. Antiamoebic and phytochemical screening of some Congolese medicinal plants. *J. Ethnopharmacol*., 61: 57-65.

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Original Article

Chemosystematics evaluation of *Eugenia* species based on molecular marker tools of flavonoids constituents

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Abstract

The chemosystematic study of nine plants species of *E. argentea, E. calcadensis, E. caryophyllata, E. discifera E. floccose, E. indica, E. rottleriana, E. singampattiana* and *E. thwaitesii* were evaluated . The present results was tentative identify the chemosystematic marker of flavonoids detected in the *Eugenia* species such as *E. argentea, E. calcadensis, E. caryophyllata, E. discifera E. floccose, E. indica, E. rottleriana, E. singampattiana* and *E. thwaitesii*. The results was first time reported in the chromatographic identification of five known flavonoids constituents were rutin, hyperoside, isoquercitrin, narcissin, and isorhamnetin found in most of the *Eugenia* species.

Keywords: Myrtaceae, *Eugenia* species, flavonoids, rutin, hyperoside

Introduction

Phytochemicals are used for chemosystematic studies. These include the primary metabolites, the secondary metabolites that are those not involved in basic metabolism of the cell, and the semantides, which are information-carrying molecules such as DNA, RNA, and proteins. Sometimes, another categorization of the systematic markers can also be defined, where the primary and secondary metabolites are referred to as micromolecules and the semantides along with the larger polysaccharides as macromolecules. This latter classification has lead to the division of chemical systematics itself into two entities. Thus chemosystematics, in the widest sense of the word, involves both micromolecules and macromolecules as systematic markers. However, very often, the terms are restricted to micromolecular systematics, involving primary and more often the secondary metabolites.

Phenolic compounds have been proved to be the most popular type of secondary metabolite (Smith,1976), and the number of chemosystematic studies based on these markers have been extensively reported. The main reason for their popularity is their quick and simple extraction from plant material. Also they are relatively easy to separate by chromatography, and are readily identified by location reagents (Smith,1976). Approximately 4000 different flavonoids have been isolated from plants. This sub class name is derived from the flavan (2-phenylchroman) skeleton which gives rise to a number of structural variations. The flavonoid group includes chalcones, aurones, flavanones, flavones, isoflavones, flaveronois, flavonols, flavan-3,4-diols (leucoanthocyanidins), flavan-3-ols (catechins) and anthocyanidins. Flavonoids have been the most successfully used for chemosystematic markers. Several examples, where chemosystematic investigations have been based on flavonoids have been reported. These include the flavonoid survey of five genera of the Calyceraceae (Bohm *et al*., 1995), the study of the flavonoids of Bignoniaceae from the "cerrado" and their taxonomic significance (Blatt *et al*. 1998), the work carried out on *Podalyriae* and *Lipariae* tribes based on seed flavonoids (De Nysschen *et al*., 1998), and the study of the distribution and chemotaxonomic significance of flavonoids in *Aloe* (Viljoen *et al*., 1998).

Harborne (1973) qualified flavonoids as being probably the most useful class of secondary plant constituents from a systematic point of view. Over the last 30 years, flavonoids have proved to be determinant at all levels of plant taxonomy (Van Sumere *et al.,* 1993). On this basis, whole plant families have been included in or excluded from specific orders. It has also been possible to assign particular flavonoid patterns at the family and species level (Harborne,1973). Flavonoids have even proved to be significant in the identification of natural plant hybrids, such as *Baptisia* (Harborne,1973) and the recognition of plant cultivars such as *Azalea* and hops (Van Sumere *et al*.,1993).
Flavonoids show enormous structural variation. They can be divided into a dozen of subclasses,

each of them varying in the degree of hydroxylation, methylation and glycosylation. Consequently flavonoids provide at least as many scorable characters as any other group of secondary substances. They also have the advantage to be more widely distributed than most other secondary substances. Flavonoids occur universally in angiosperms, gymnosperms and pteridophytes and therefore their use as chemosystematic markers is not restricted. In addition flavonoids seem to be amongst the most stable chemical characters in plants. Qualitative variation at the species level is very limited. Moreover, when it comes to ease and speed of identification, flavonoids are again highly rated (Harborne, 1967).

Myrtaceae consists of around 129 genera and 4620 species (Mabberley,1997). One important member of this family is *Eugenia*, which is one of the larger genera with around 500 species (Mabberley,1997). In the present study was chemosystematic investigation on *Eugenia argentea, E. calcadensis, E. caryophyllata, E. discifera, E. floccose, E. indica, E. rottleriana, E. singampattiana* and *E. thwaitesii* based on chemical constituents of flavonoids.

Methods and Materials

Extract preparation

100g of air-dried and powdered plant material of *Eugenia argentea, E. calcadensis, E. caryophyllata, E. discifera, E. floccose, E. indica, E. rottleriana, E. singampattiana* and *E. thwaitesii* were separately extracted with 50% ethanol under reflux. After the removal of ethanol *in vacuo,* the aqueous residue was consecutively treated with chloroform and ethyl acetate. Ethyl acetate extract was evaporated to dryness to give a solid residue and examined by TLC. Flavonoids were separated in Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26) and visualized by 1% 2-aminoethyldiphenyl borinate solution in methanol, followed by 5% polyethylene glycol 4000 in absolute ethanol at 365 nm (Wagner and Bladt, 1996).

Quantitative determination of flavonoids

The content of flavonoids in ethyl acetate extract, calculated as hyperoside, was determined by spectrophotometry at 320nm (*European Pharmacopoeia*, 2002).

Results and Discussion

Listed table - 1 gave the Rf values and the tentative identities of flavonoids detected in nine *Eugenia* species such as *E. argentea, E. calcadensis, E. caryophyllata, E. discifera E. floccose, E. indica, E. rottleriana, E. singampattiana* and *E. thwaitesii*. Chromatographic identification of five known flavonoids was namely rutin, hyperoside, isoquercitrin, narcissin, and isorhamnetin found in most of the *Eugenia* species (Table-1).

Compound of Rf value 0.98 was found in nine *Eugenia* species, but *Eugenia caryophyllata,* and *Eugenia indica* was very prominent detected. Compound of Rf value 0.89 was found in nine *Eugenia* species, but *Eugenia argentea* and *discifera* was observed very prominent. Compound of Rf = 0.78 was found in 8 *Eugenia* species, but *Eugenia floccosa* was very prominent detected and did not detected *Eugenia calcadensis.* Compound of Rf value 0.72 was found in seven *Eugenia* species, but *Eugenia indica,* and *Eugenia singampattiana* were very prominent detected in flavonoids. *Eugenia discifera* and *E. thwaitesii* were not detected. Compound of Rf value 0.65 was found in seven *Eugenia* species, but *Eugenia caryophyllata* was very prominent detected in flavonoids. Earlier reported that chromatographic profile of *E. elliptica* seems to be less complex in terms of flavonoid composition compared to the 3 other *Eugenia* species. This difference seems to be in accordance with morphological data. Leaves of *E. elliptica,* for instance, are larger than those of *E. pollicina, E.fasciculata,* and *E. orbiculata* which are themselves almost similar in size. Also leaves of *E. elliptica* are acute at the apex, while those of *E. pollicina, E, fasciculata,* and *E. orbiculata* have obtuse apices. More often, leaves of *E. elliptica* have more than 10 pairs of lateral veins, whereas those of the 3 other species usually have less (Bosser, 1987).

The results of the flavonoids contents are shown in the Fig. 1. A marked variability in the flavonoids content of *Eugenia* species has been reported. These species are variability content may be attributed to the origin or the vegetative stage and this study might be interesting from a taxonomically identification point view of confusion of species.

Table-1: Distribution of flavonoids constituents of ethyl acetate extracts of *Eugenia* species

Species	Flavonoid detected				
	Rf = 0.98	Rf = 0.89	Rf = 0.78	Rf = 0.72	Rf = 0.65
Eugenia argentea	+	+++	+	+	++
Eugenia calcadensis	++	+	-	++	+
Eugenia caryophyllata	+++	++	+	+	+++
Eugenia discifera	+	+++	++	-	-
Eugenia floccosa	+	+	+++	+	++
Eugenia indica	+++	++	+	+++	-
Eugenia rottleriana	++	+	++	+	+
Eugenia singampattiana	+	++	++	+++	++
Eugenia thwaitesii	++	+	+	-	+

[+++: Very prominent; ++: Prominent; +: Present; (+): Trace; -: Not detected].

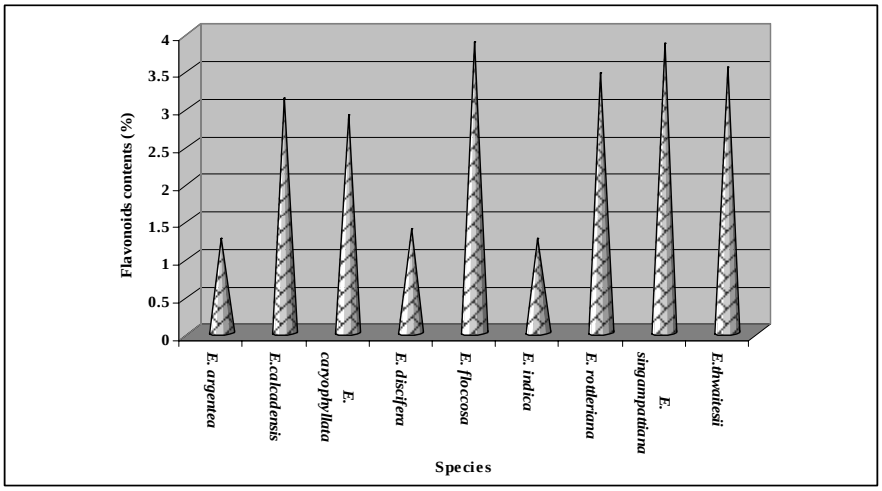

Fig -1: Flavonoids contents of *Eugenia* species

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References

Bosser, J., Cadet Th, Gueho, J. and Marais, W.1987. *Flore des Mascareignes Réunion,Maurice, Rodrigues*. The Sugar Industry Research Institute, Mauritius; Loffice de la recherchescientifique et technique outremer, Paris; *The Royal Botanical Garden, Kew*, 53(6): 11-19.

Harborne, J.B.1967. *Comparative Biochemistry of the Flavonoids*. London, Academic Press, Inc. Ltd.

Wagner, H. and Bladt, S.1996. *A Thin Layer Chromatography Atlas. Plant Drug Analysis*. 2ndEdition. New York, Springer-Verlag.

Lai Fang, N.R., Bahorun, T. and Khittoo, G.2001. Chemosystematics: A new source of evidence for the classification of the endemic flora of Mauritius. *AMAS*, Food and Agricultural Research Council, Réduit, Mauritius.73-79.

European Pharmacopoeia,2002. 4th ed., Council of Europe, Strasbourg,742–743.

Mabberley, D.J.1997. The Plant-book. Cambridge University Press, Cambridge, UK.

Smith, PM. 1976. *The chemotaxonomy of plants*. United Kingdom, Edward Arnold (Publishers) Ltd.

Blatt, C.T.T., Dos Santos, M.D. and Salatino, A. 1998. Flavonoids of *Bignoniaceae* from the "cerrado" and their possible taxonomic significance. *Plant Systematics and Evolution* 210: 289-292.

De Nysschen, A., Van wyk, B.E. and Van Heerden, F.R. 1998. Seed flavonoids of the

Podalyrieae and *Liparieae* (*Fabaceae*). *Plant Systematics and Evolution* 212: 1-11.

Viljoen, A.M., Van Wyk, B.E. and Van Heerden, F.R. 1998. Distribution and chemotaxonomicsignificance of flavonoids in *Aloe* (*Asphodelaceae*). *Plant Systematics and Evolution* 211: 31-42.

Harborne, J.B. 1973. Flavonoids as systematic markers in the angiosperms. p 103-115. In Bendz, G and Santesson, J. eds. *Chemistry in Botanical Classification.* Proceedings of the Twenty-Fith Nobel Symposium, Sweden. New York and London, Academic Press.

Van Sumere, C.F., De Cooman, L., Everaert, E., De Keukeleire, D. and Vande Casteele, K. 1993. Phenolic markers in chemotaxonomy and plant cultivar recognition. P 257- 266. In Scalbert, A. ed. *Polyphenolic Phenomena* . Paris, INRA editions.

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Original Article

In vitro propagation of wild yam, *Dioscorea wightii* through nodal cultures

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Abstract

In vitro culture of *Dioscorea wightii* belongs to the family Dioscoreace. An efficient micropropagation protocol was developed for the medicinal plant *Dioscorea wightii* using nodal segments for axillary shoot proliferation. Callus initiation was stimulated at the basis of the explants in 100% of the cultures by 0.15-1.75µM BA, 0.75-5.0µM Kinetin, 0.15-0.30µM 2iP.The average fresh mass of calluses produced under these latter conditions was significantly promoted by increased concentrations, and BA at 1.75µM supported the highest callus fresh mass per explant, *ca.* 253mg. Shoots were produced in 100% of the cultures by all growth regulators tested BA, 2iP, and kinetin. The optimized micropropagation, callus, shoot and root culture protocols offer the possibility to use cell/organ culture techniques for vegetative propagation and secondary metabolism studies.

Key words: *Dioscorea wightii;* Dioscoreace; tuber; Micropropagation

Introduction

About 85% of traditional medicines involve the use of plant extracts (Vieira and Skorupa,1993). Many plant species, possessing medicinally important compounds, are disappearing at an alarming rate due to destruction of its natural habitats owning to rapid agricultural development, urbanization, indiscriminate deforestation and uncontrolled collection of plant materials. Plant Tissue culture from the back bone of plant biotechnology, i.e micropropagation, induction of somoclones, somatic hybridization, and cryopreservation has play an important role.

The genus *Dioscorea* includes over 600 species (Ayensu,1972), and is of considerable economic importance. A number of *Dioscorea* wild species are the source of compounds used in the synthesis of sex hormones and corticosteroids and cultivated species are the source of food in some tropical countries (Coursey, 1976). These true yams are the source of agents used to treat such varied conditions as inflammation, joint pain, diabetes, infections and dysmenorrhea. The pharmacologically active components of the *Dioscorea* species include diosgenin, which is a steroidal saponin, and dioscin, a form of diosgenin with sugars attached (Ramberg and Nugent, 2002).

In-Vitro conservation of *Dioscorea* species has been achieved using nodal cuttings (Chaturvedi,1975; Lakshmisita *et al*., 1976; Mantell *et al*.,1978; Alizadeh *et al*., 1998; Yan *et al*. 2002; Chen *et al*.,2003), bulbils (Asokan *et al*.1983), zygotic embryos (Viana and Mantell, 1989), meristem tips (Malaurie *et al*.,1995), immature leaves (Kohmura *et al*., 1995) and roots (Twyford and Mantell, 1996). Attention has been paid to the clonal propagation through *in vitro* production of microtubers in *D. abyssinica* (Martine and Cappadocia, 1991), *D. alata* (Mantell and Hugo,1989; Martine and Cappadocia, 1991; John *et al*.,1993; Jasik and Mantell, 2000), *D. batatas* (Koda and Kikuta, 1991), *D. composite* (Alizadeh *et al*., 1998) and *D. floribunda* (Sengupta *et al*., 1984). The present investigation was carried out to see the effects of *in vitro* culture conditions MS medium containing cytokinins such as BA, kinetin and 2iP on nodal culture on *Dioscorea wightii.*

Materials and Methods

Explants of node of 2-5 mm in length were sterilized by the standardized method and cultured for 60 days on the MS medium supplemented with various concentrations 0.15-5.0mg/l of three plant hormones, BA and kinetin and 2iP. For the nodal segment culture, nodal segments with a single node were collected from vines of plants grown in the same greenhouse and sterilized in the same method as the shoot-

apex culture. Stem segments with a single node about 2 cm long were transplanted on solid MS media and cultured at 25°C and 16hr illumination.

Results and Discussion

The effects of cytokinins on morphogenesis of nodal segment explants are presented in tables 1.

Table -1: The frequency (%) of morphogenic responses and callus fresh mass of nodal segment explants of *Dioscorea wightii* after 50 days on medium containing cytokinins.

Growth regulators	Concentration (µM)	Shoots (%)	Roots (%)	Callus (%)	Normal plantlets	Callus fresh mass (mg)
BA	0.00	100	100	0	100	-
	0.15	100	100	100	0	95.63
	0.30	100	52	100	4	125.31
	1.75	100	15	100	5	253.12
	2.25	100	0	100	0	115.78
	5.00	100	0	0	0	12.25
Kinetin	0.00	100	100	0	100	-
	0.15	100	98	0	100	-
	0.30	100	90	0	100	42.23
	1.75	100	85	90	0	96.32
	2.25	100	70	100	0	78.25
	5.00	100	60	100	0	110.25
2iP	0.00	100	100	0	100	-
	0.15	100	95	100	0	95.32
	0.30	100	96	100	0	161.32
	1.75	100	36	90	0	175.78
	2.25	100	25	85	0	135.14
	5.00	100	0	80	0	148.36

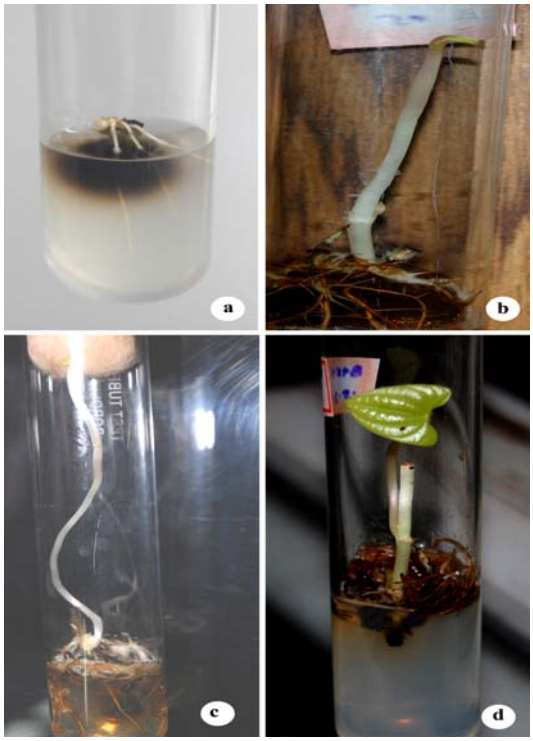

Fig.1: *In vitro* micropropagation of *D.wightii*

Callus initiation was stimulated at the basis of the explants in 100% of the cultures by 0.15-1.75µM BA, 0.75-5.0µM Kinetin, 0.15-0.30µM 2iP.The average fresh mass of calluses produced under these latter conditions was significantly promoted by increased concentrations, and BA at 1.75µM supported the highest callus fresh mass per explant, *ca*. 253mg. Shoots were produced in 100% of the cultures by all growth regulators tested BA, 2iP, and kinetin. 100% plantlets of cultures grown on 0.15-0.30µM kinetin and control.

Growth inhibitory effect of kinetin on shoot numbers of *D. oppositifolia* and *D. pentaphylla* microplants was observed. Lakshmisita *et al*. (1976), however, reported that the kinetin supplied at either 11.6 or 46.4 µM significantly increased the shoot development in *D. floribunda* shoots cultures. Also the promotive effects of kinetin (46.4µM) on plantlet growth for *D. bulbifera*, which increased the number of shoots per plantlet. The present study successfully developed in rapid protocol obtained *in vitro* by nodal segment culture. The tissue culture technique is expected to develop an effective system of large scale production of *Dioscorea wightii*.

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References

Ayensu, E.S.1972. Anatomy of the monocotyledons VI Dioscoreales Oxford Press, Oxford.p.82.

Coursey, D.G.1976. Yams, *Dioscorea* spp. (Dioscoreaceae) In: Simmonds ED (Ed) Evolution of crop plants. Longman, London.pp.70-74.

Coursey, D.G., 1967. Yams. Longman, Green and Co, London, 230p.

Forsyth, C. and Van Staden,J.1982. An improved method of *in vitro* propagation of *Dioscorea bulbifera*. *Plant Cell Tissue Organ Cult*., 1: 275-28112.

Ramberg, J.M.S. and Nugent, S. 2002. History and uses of *Dioscorea* as a Food and Herbal Medicine. Glycosci. Nutr., 3(6): 1-5.

Chaturvedi, H,C.1975. Propagation of *Dioscorea floribunda in vitro* culture of single node segments. *Curr. Sci*., 44: 839-841.

Mantell, S.H., Haque, S.Q. and Whithall, A.P.1978. Clonal propagation of *Dioscorea alata* L. and *Dioscorea rotundata* Poir yams by tissue culture. *J. Hortic. Sci*., 51: 95-98.

Alizadeh, S., Mantell, S.H. and Viana, A.M. 1998. *In vitro* shoot culture and microtuber induction in the steroidal yam *Dioscorea composite* Hemsl. *Plant Cell Tissue Organ Cult*., 53: 107-112.

Chen, Y., Fan, J., Yi, F., Luo, Z. and Fu, Y. 2003. Rapid clonal propagation of *Dioscorea zingiberensis*. *Plant Cell Tissue Organ Cult*. 73: 75-80.

Yan, Y.C., Lin Hh, Q.I. Dai, and Q.Q.Huang, 2002. Studies on tissue culture and rapid propagation of *Dioscorea zingiberensis*. J Scichuan University (Natural Science Edition) 39: 136-140.

Asokan, M.P., O'Hair, S.K. and, Litz,1983. In *vitro* plant development from bulbil explants of two *Dioscorea* species. *Hortic. Sci*.,18:702-703.

Viana, A.M., and Mantell, S.H.1989. Callus induction and plant regeneration from excised zygotic embryos of the seed propagated yams *Dioscorea composita* hemsl. and *D.cayenensis* Lam. *Plant Cell Tissue Organ Cult*.,16: 113-122.

Malaurie, B., Pungu, O. and Trouslot,M.F. 1995b. Effects of growth regulators concentrations on morphological development of meristem-tips in *Dioscorea cayenensis, D .rotundata* complex and *D. praehensilis*. *Plant Cell Tiss. Org. Cult*., 42: 215-21.

Kohmura, H., Araki, H. and Imoto, M. 1995. Micropropagation of 'yamatoimo' Chinese Yam (*Dioscorea opposita*) from immature leaves. *Plant Cell Tissue Organ Cult*.. 40: 271-276.

Twyford, C.T. and Mantell, S.H. 1996. Production of somatic embryos and plantlets from root cells of Greater yam. *Plant Cell Tissue Organ Cult*.. 46: 17-26.

Martine, J. and Cappadocia, M.1991. *In vitro* tuberization in *Dioscorea alata* L. 'Brazo fuerte' and 'Florido' and *D. abyssinica* Hoch. *Plant Cell Tissue Organ Cult*.. 26: 147-152.

Mantell,S.H. and Hugo, S.A.1989. Effects of photoperiod, mineral medium strength, inorganic ammonium, sucrose and cytokinin on root, shoot and microtuber development in shoot cultures of *Dioscorea alata* L. and *D. bulbifera* L. Yams. *Plant Cell Tissue Organ Cult*.. 16: 23-37.

Jasik,A. and Mantell, S.H.2000. Effects of jasmonic acid and its methylester on *in vitro* microtuberisation of three food yam (*Dioscorea*) species. *Plant Cell Rep*., 19: 863-867.

John, J.L., Courtney, W.H. and Decoteau, D.R. 1993. The influence of plant growth regulators and light on microtuber induction and formation in *Dioscorea alata* L. cultures. *Plant Cell Tissue Organ Cult*.. 34: 245-252.

Koda, Y. and Kikuta, Y. 1991. Possible involvement of jasmonic acid in tuberization of yam plants. *Plant Cell Physiol*., 32: 629-633.

Sengupta, J., Mitra, G.C. and Sharma, A.K. 1984. Organogenesis and tuberization in cultures of *Dioscorea floribunda*. *Plant Cell Tissue Organ Cult*.. 3:325-331.

Vieira, R.F. and Skorupa, L.A. 1993. Brazilian medicinal plants gene bank. *Acta Hort*.,330: 51-58.

Poornima, G. N. and Ravishankar Rai, V. 2007. *In vitro* propagation of wild yams, *Dioscorea oppositifolia* (Linn) and *Dioscorea pentaphylla* (Linn). *African Journal of Biotechnology*,6(20): 2348-2352.

Uduebo, A.E.1971. Effect of external supply of growth substances on axillary proliferation and development in *Dioscorea bulbifera*. *Ann. Bot*., 35: 159-163.

Lakshmisita, G., Bammi, R.K. and Randhawa, G.S.1976. Clonal propagation of *Dioscorea floribunda* by tissue culture. *J. Hortic. Sci*.,51: 551-554.

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Review

An inside preview of Ethnopharmacology of *Cissampelos pareira* Linn.

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Abstract

Cissampelos pareira is significant medicinal plant of herbal materia medica. It is used in the treatment of wide range of diseases in Traditional Chinese Medicine, Ayurevda and western herbalism. The plant abounds in isoquinoline alkaloids; the chemicals that received a great deal of attention and research in the late 1960. Antitumour potential of cissampareine and neuromuscular blocking effects of hayatine are of special interest. The review summarizes ethno pharmacological investigations carried out on the plant with special reference to isoquinoline alkaloids.

Keywords: *Cissampelos pareira; isoquinoline alkaloids; pelosine; cissampareine; hayatine; pharmacology*

Introduction

Cissampelos pareira Linn. is significant plant of family Menispermaceae. There are 37 plant species summarized under this botanical name. Their taxonomic position is not clear. In most cases, *C. pareira* or Pareira is used and the single species are called subspecies. It is found in subtropical parts of India, Asia, East Africa and America (Singh,2005).

The plant is a climbing shrub, 2 - 5m high with a thickened root. Leaves have an orbicular shape 7-14 cm in diameter. They are membranous or leathery, veined, glabrous to densely pilose. Flowers are green, male ones in short umbels, 10 - 12cm long, females in pendulous spikes, 7 - 10cm long, with a little round leaflet at the base of every flower (Prasad *et al*.,1962; Smitin and Larsen,1991).

Traditional medicinal use

Brazil*: C. pariera* is widely employed in herbal medicine today as a diuretic and as a tonic, as well as to reduce fever and relieve pain. It is often employed for menstrual cramps, difficult menstruation, excessive bleeding and uterine hemorrhages, fibroid tumors, pre- and postnatal pain, colic, constipation, poor digestion, and dyspepsia (Mukerji and Bhandari,1959; Feng *et al*.,1962).

French Guyana*:* The roots are used in the treatment of dysuria and renal calculi. The Wayãpi Indians use a decoction of the leaf and stem as an oral analgesic (Gogte,2000).

India: In Ayurvedic system of medicine, the leaves are used in the treatment of indolent ulcers (Kirtikar and Basu,2001) and diarrhea (Amresh *et al*.,2003). The plant is considered to be antiseptic and on account of this property, it is used in the treatment of urinary tract infection (Dandiya and Chopra,1970). Expressed juice of *C. pareira* is given in migraine (Singh,2005).

Mexico: *C. pariera* has a long history of use for muscle inflammation, snakebite, rheumatism, diarrhea, dysentery, and menstrual problems (Mokkhasmit,1971).

South America*: C. pariera* is commonly referred to as the midwives' herb throughout South America. It ha been used for all types of women's ailments. The root is used in tropical countries to prevent a threatened miscarriage and to stop uterine hemorrhages after childbirth. Midwives in the Amazon still carry abuta with them for menstrual cramps and pre- and postnatal pain, excessive menstrual bleeding, and uterine hemorrhaging (Floriani,1936).

Thailand: The extract from its leaves can be used to make gel. The dark green gel is used as medicine for treating fever in local people. Local people use this plant as a diuretic and for the treatment of a variety of ailments, including

asthma and for traumas (Mokkhasmit *et al*.,1971).

Phytochemistry: An amorphous, white alkaloid, *pelosine* (Figure 1) was studied in association with an indifferent body, *deyamittin*. Cissampelosine was reported from *C. pariera* which was later on shortened as pelosine (Wiggers, 1838).

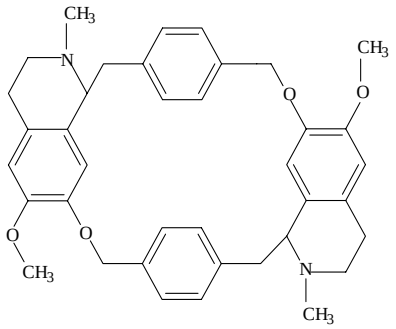


Fig.1: Structure of Pelosine

Eleven years later, studies on pelosine and bebeerine were undertaken and both the alkaloids were proved to be different (Bodeker,1849). However a study proved similarity with the alkaloids *bebeerine* and *buxine* (Flückiger,1869). A comparative analysis of *C. pareira* demonstrated presence of starch, gum, tannin, phlobaphene, and an alkaloid (Ringer and Brooke, 1982).

C. pareira contain a group of plant chemicals called isoquinoline alkaloids (Roy *et al*.,1959). Since the late 1960s, these chemicals have received a great deal of attention and research (Boissier *et al*.,1965). Cissampareine (Figure 2) was reported from *C. pareira* growing in Peru (Kupchan,1964). Cissampareine was found to show significant and reproducible inhibitory activity against human carcinoma of the nasopharynx carried in cell culture (KB). Cissampareine is isomeric with methylwarifteine found in dried rhizomes of *C. ovalifolia* DC.

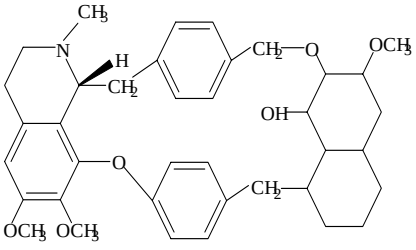


Fig. 2: Structure of Cissampareine

(++)-4^{II}-*O*-methylcurine (Fig. 3), a new alkaloid was isolated from *C. pareira* (Haynes *et al*.,1966). *l*-curine (Fig.4), *d*-iso-chondrodendrine (Fig.5), and hayatine (Fig.6) were isolated from the roots and vines of *C. pareira* from Madras (Kupchan *et al*.,1966). Preliminary pharmacological study of the methanol-extractable alkaloids, of the methiodide prepared from the latter mixture, and of the quaternary alkaloids, showed that all had curare-like activity (Mukerji and Bhandari,1959).

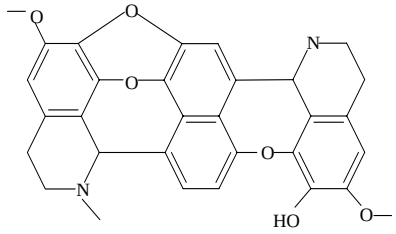


Fig. 3: Structure of (++)-4”-O-Methylcurine

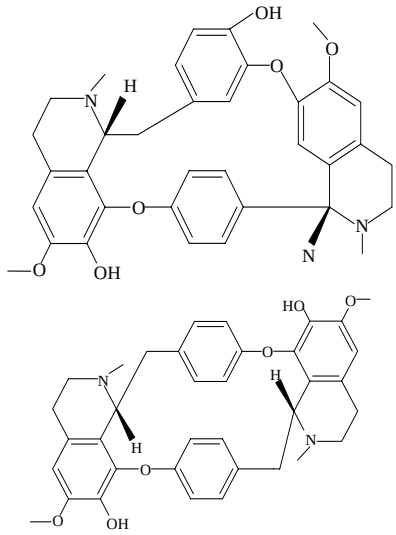


Fig 4: Structure of *l*-curine
Fig .5: Structure of *d*-iso-chondrodendrine

A study reported stereochemistry and pharmacology of hayatine (Fig.6) (Sur, and Pradhan,1963). Chemical investigation on the roots from Kashmir, reported 0.33 % of alkaloids, mainly hayatine and bebeerines (Kirtikar and Basu,2001), 0.2 % essential oils, 3.4 % fixed oils and a sterol (Bhattacharji, 1952). In the same year, stereochemistry of hayatidine (Fig.8) and hayatinine (Fig.12) was reported (Bhatnagar *et al*.,1967; Bhatnagar and Popli,1967).

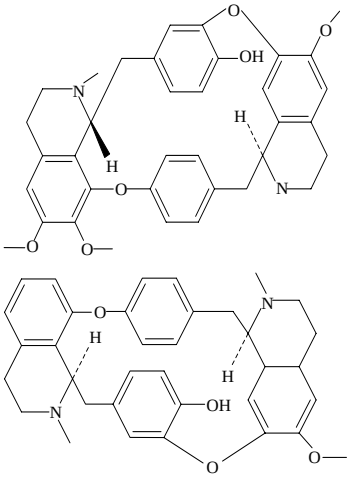


Fig. 6: Structure of Hayatine
Fig .7: Structure of Bebeerine

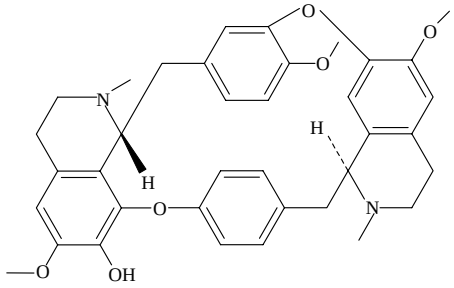


Fig .8: Structure of Hayatidine

Cissamine (Fig.9) and cycleanine (Fig.10) have been reported from the roots (Anwer et al.,1968; Bhattacharji, *et al*.,1952). Root is reported to contain *l*-curine. Root bark is reported to contain menismine, pareirine (Fig.11) and hayatinine (Combes *et al*.,1965; Dwuma-Badu *et al*.,1975).

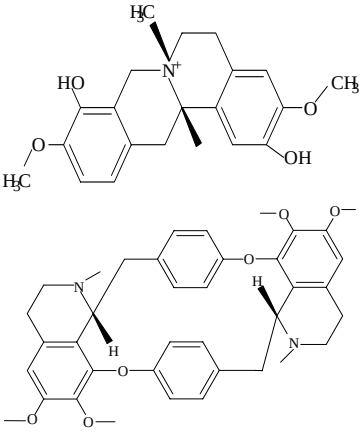


Fig.9: Structure of Cissamine
Fig. 10: Structure of Cycleanine

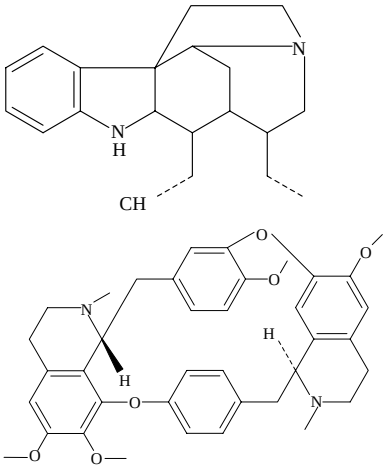


Fig. 11: Structure of Pareirine
Fig .12: Structure of Hayatinine

Tetrandrine (Fig.13) has been reported from the roots of *C. pareira* growing in Thailand (Rojanasonthorn,1970). Dicentrine (Fig.14), dihydrodicentrine, cycleanine, insularine (Fig.15) and isochondrodendrine have been reported from roots of the plant growing in Ghana (30). Isolation of pareirubrine A (Fig.16) and B (Fig. 17), novel tropoloisoquinoline alkaloids with antileukemic activity has been reported (Morita *et al*.,1993).

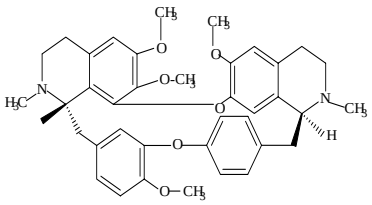


Fig. 13: Structure of Tetrandrine

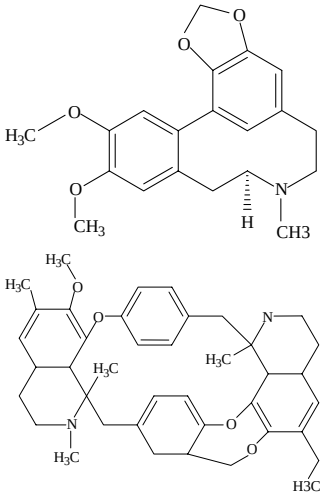


Fig 14. Structure of Dicentrine
Fig 15. Structure of Insularine

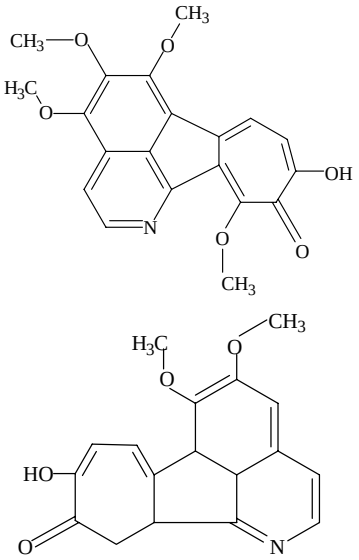


Fig. 16: Structure of Pareirubrine A
Fig. 17: Structure of Pareirubrine B

Tropoloisoquinoline alkaloid pareitropone (Fig. 18) has been reported (Morita *et al*.,1993). A novel azafluoranthene alkaloid, norimeluteine (Fig.19), has been isolated as a cytotoxic substance from *C. pareira* together with an alkaloid having the same skeleton, norruffscine (Morita *et al*.,2002).

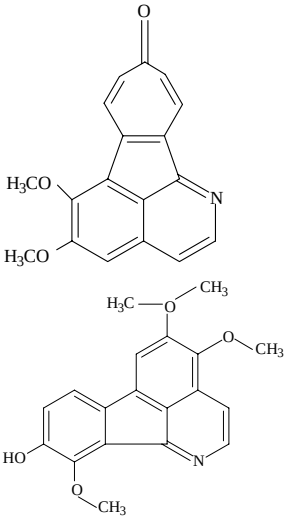


Fig.18:Structure of Pareitropone
Fig. 19. Structure of Norimeluteine

An antiprotozoal chalcone-flavone dimer, cissampeloflavone (Fig.21) has been isolated from the aerial parts of *C. pareira*. It has good activity against *Trypanosoma cruzi* and *T. brucei rhodesiense* and has a low toxicity to the human KB cell line (Carabot *et al*.,2003). D-Qurecitol (Fig.22) and grandirubrine (Fig.23) have been reported.

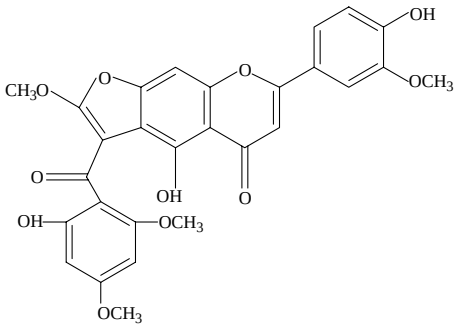
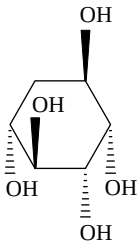


Fig. 21: Structure of Cissampeloflavone
Fig .22: Structure of D-Qurecitol



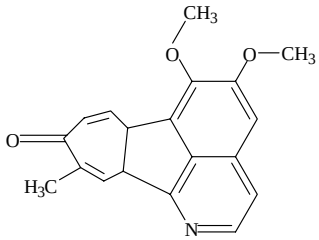


Fig. 23: Structure of grandirubrine

Pharmacology

Antinociceptive and antiarthritic activity: In the present study, 50% aqueous ethanolic extract of roots of *C. pareira* at the dose levels of 100–400 mg/kg, once daily for 3 days exhibited significant ($P < 0.001$) resistance against mechanical pain after 30 min in analgesymeter induced pain in mice. In acetic acid (0.6%; i.p.) inducing writhing, *C. pareira* significantly ($P < 0.05$) decreased the writhing episodes; the degree of percent protection at 200 and 400 mg/kg was 22.73 and 51.63. The hot plate reaction time was increased by 2.07 ($P < 0.05$) and 2.70 ($P < 0.001$) folds, respectively. Further *C. pareira* showed the dose dependent significant protective effect against complete Freund's adjuvant induced arthritis (Amresh *et al*.,2001).

Anti-inflammatory activity: Oral administration of 50% ethanolic extract of the aerial part of *C. pareira* exhibited significant and

dose dependent anti-inflammatory activity in the carrageenin test, which was based on interference with prostaglandin synthesis, as confirmed by the arachidonic acid test. In the abdominal writhing test induced by acetic acid, higher dose of the plant extract had the highest analgesic activity, whereas in the hot-plate test the best dose was 100 mg/kg ($p< 0.05$). The LD_{50} showed that *C. pareira* (2000 mg/kg) presented low toxicity (Amresh *et al*.,2007).

In yet another study, 50% ethanolic extract of *C. pareira* roots in acute, subacute and chronic models of inflammation was assessed in rats. *Per os* (p.o.) administration of *C. pareira* (200, 400 mg/kg) exhibited significant anti-inflammatory activity. In acute inflammation as produced by carrageenin 59.55% and 64.04%, by histamine 15.38% and 30.77%, by 5-hydroxytryptamine 17.78% and 31.11% and by prostaglandin E_2-induced hind paw edema 19.23% and 30.77% protection was observed. While in subacute anti-inflammatory models using formaldehyde-induced hind paw edema (after 1.5 h) 38.36% and 47.95% and in chronic anti-inflammatory model using cotton pellet granuloma 15.02% and 19.19% protection from inflammation was observed (Amresh *et al*.,2008).

Antifertility activity: *C. pareira* leaf extract, when administered orally, altered the estrous cycle pattern in female mice, prolonged the length of estrous cycle with significant increase in the duration of diestrus stage and reduced significantly the number of litters in albino mice. The analysis of the principal hormones involved in estrous cycle regulation showed that the plant extract altered gonadotropin release (LH, FSH and prolactin) and estradiol secretion. The oral LD50 of the extract was found to be 7.3 g/kg in mice. (Ganguly *et al*.,2007)

Antioxidant activity*: C. pareira* extract showed significant antioxidant activity in the 1,1 - diphenyl-2-picrylhydrazyl assay. *C. pareira* extract was found to significantly scavenge superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide at a dose regimen of 50 to 400 µg/kg *in vitro*. *C. pareira* extract also inhibited hydroxyl radical-induced oxidation of proteins *in vitro*. *C. pareira* extract exhibit a potent protective activity in an acute oxidative tissue injury animal model: benzo (a) pyrene-induced gastric toxicity in mice *in vivo* (Amresh et al.,2007).

Chemo preventive effects*:* The protective effect of *C. pareira* extract was studied against benzo (*a*) pyrene [B(*a*)P]-induced gastric cancer in mice, and the tumor incidence was reduced and the mean number of tumors and the tumor multiplicity were reduced significantly and dose-dependently. The modulatory effect of *C. pareira* extract was also examined on carcinogen metabolizing phase I and phase II enzymes, antioxidant enzymes, glutathione content, lactate dehydrogenase, and lipid peroxidation in liver. Significant increases in the levels of acid-soluble sulfhydryl (-SH) and cytochrome P_{450} contents and in enzyme activities of cytochrome P_{450} reductase, cytochrome b_5 reductase, GST, DTD, SOD, catalase, glutathione (GSH) peroxidase, and GSH reductase but decreased malondialdehyde (MDA) were observed. (Amresh *et al*.,2007)

Anti-hemorrhagic effects*:* To establish the anti-hemorrhagic activity of aqueous extract from leaves of *C. pareira*, the skin of mice was injected with a mixture of extract and venom, and it as found that extract produced a total inhibition of this activity. On the other hand, experiments regarding the anti-proteolytic activity were conducted observing the effect on casein in a test tube or on biotinylated casein in a microplate. None of the two procedures was able to show any inhibitory activity (Badilla *et al*.,2008).

Toxicity

In the acute toxicity test, oral administration of 2 g/kg of *C. pareira* produced neither mortality nor changes in behavior or any other physiological activities in mice. In subacute toxicity studies, no mortality was observed when the two doses of 1 or 2 g/kg day of 50% aqueous ethanolic extract of *C. pareira* were administered p.o. for a period of 28 days in rats. There were no significant changes occurred in the blood chemistry analysis in both sexes of animals. Hematological analysis showed no marked differences in any of the parameters examined in either the control or treated group of both sexes. Pathologically, neither gross abnormalities nor histopathological changes were observed (Amresh *et al*.,2008).

Pharmacology of hayatine

Hayatin methiodide has been used as a muscle relaxant during surgery in 100 patients. This drug provided adequate relaxation for

endotracheal intubation and surgery. It appeared to be about one-third as potent as tubocurarine. The duration of both these drugs was of equal magnitude in equipotent doses. The neuromuscular block produced by this drug could be completely reversed by neostigmine. It was relatively free from serious side-effects and appears to be a promising muscle relaxant.

References

Singh, A.P., 2005. *Dravyaguna Vijnana*. Gupta, A. Chaukhambha Orientalia, New Delhi, pp.29-30.

Prasad, S., Gupta, K.C., and Bhattacharya, L.C., 1962. Pharmacognostical study of *Cissampelos pareira* Linn. *J. Sci. and Indust. Res*., 21:150-154.

Smitin, T., and Larsen K., 1991. *Flora of Thailand*. Vol.5 Part 3. Bangkok: The Forest Herbarium, Royal Forest Department, pp.23.

Mukerji, B., and Bhandari, P.R., 1959. *Cissampelos pareira* source of a new curariform drug. *Planta. Med*., 7:250-259.

Feng PC., *et al*., 1962. Pharmacological screening of some West Indian medicinal plants. *J. Pharm. Pharmacol.*, 14: 556–61.

Gogte., V.M., 2000. *Ayurvedic pharmacology and therapeutic uses of medicinal plants* (*Dravyagunavigyan*), First ed., Bharatiya Vidya Bhavan (SPARC), Mumbai Publications, pp. 421-422.

Kirtikar, K.R., and Basu, B.D., 2001. *Indian Medicinal Plants*. Vol. I, 2nd ed., Oriental Enterprises, Dehradun, India, pp. 131-134.

Amresh, G., Rao, V., Mehrotra, S., and Shirwaikar, A., 2003. *Standardization and ethnopharmacological evaluation of antidiarrhoeal herbal formulation*. Manipal Academy of Higher Education, Manipal (Dissertation), Karnataka, India, pp. 24-40.

Dandiya, P.C., and Chopra, Y.M., 1970. *Cissampelos pareira* Linn. *Indian J. Pharm.*, 2:67.

Floriani, J., 1936. Pharmacology of *Cissampelos pareira* var Gardneri. *Rev. Farm.*, 78:49.

Mokkhasmit, M., *et al*. 1971. Pharmacological evaluation of Thai medicinal plants continued. *J. Med. Ass. Thailand.*, 54: 490–504.

Wiggers, A., 1838. Pelosine. *Annalen. Der. Pharmacie.*, 27:29.

Bodeker, C., 1849. Constitutions of pelosine and bebeerine. *Ann. Chem. Pharm*., 69:37.

Flückiger, G., 1869. *Pelosine, bebeerine* and *buxine. Ann.Chem. Pharm*., 23: 49.

Ringer, P., and Brooke, M. A., 1892. Comparative analysis of *C. pareira*. *Am. J. Pharm.*, 255.

Roy, P.K., Dutta, A.T., Ray, G.K., and Mukerji, B.,1952. A preliminary note on the pharmacological action of the total alkaloids isolated from *Cissampelos pareira* Linn. (False Pareira brava). *Med. Res.*, 40: 95-100.

Boissier, J.R., Combes, G., Pernet, R., and Dumont, C., 1965. Contribution to the study of alkaloids of some Menispermaceae of Madagascar: *Cissampelos pareira, Cyclea madagascariensis, Anisocyclea grandidieri* and *Spirospermum penduliflorum* Engl. summ. *Lloydia*., 28:191-198.

Kupchan, M.S., Patel, A.C., and Fujita, E., 1964. Tumor inhibitors VI. Cissampareine, new cytotoxic alkaloid from *Cissampelos pareira*. Cytotoxicity of bisbenzylisoquinoline alkaloids. *J. Pharm. Sci.*, 54:580- 583.

Haynes, L.J., Herbert, E.J., and Plimmer, J.R. 1966. (++)-4''-*O*-methylcurine from *C. pareira* L. *J. Chem. Soc.*, 43: 615 – 617.

Kupchan, S.M., Yokoyama, N., and Beal, J.L., 1966. Menispermaceae alkaloids I. The alkaloids of *Cissampelos pareira* Linn. and the origin of *radix pareirae bravae. J. Am. Pharm. Assoc.*, 49:727-731.

Sur, R.N., and Pradhan, S.N., 1963. Studies on Cissampelos alkaloids: L Action of hayatin derivatives on the central nervous system of cat and dogs. *Archiv .Int. Pharmacodyn. Therap.*, 152:6-14.

Bhattacharji, S., Sharma, V.N., and Dhar, M.L. 1952. Chemical examination of the roots of *Cissampelos pareira* Linn. *J.Sci. Indust. Res.*, [India] 11B: 81-2.

Bhatnagar, A.K., Bhattacharji, S., Roy, A.C., Popli, S.P., and Dhar, M.L. 1967. Chemical examination of the roots of *Cissampelos pareira* Linn. IV Structure and stereochemistry of hyatidine. *J. Org. Chem.*, 32:819-20.

Bhatnagar, A.K., and Popli, S.P., 1967. Chemical examination of the roots of *Cissampelos pareira* Linn.: III.-Structure and stereochemistry of hayatinine. *Ind.J. Chem.*,5:102-4.

Anwer F, Popli SP, Srivastva RM, Khare MP. Studies in medicinal plants 3. Protoberberine alkaloids from the roots of *Cissampelos pareira* Linn. *Experientia*. 1968; 15.

Combes, G., Pernet, R., and Dumont, C., 1965. Contribution to the study of alkaloids of some Menispermaceae of Madagascar: *Cissampelos pareira, Cyclea madagascariensis, Anisocyclea*

grandidieri and *Spirospermum penduliflorum* Engl. summ. *Lloydia.*, 28:191-8.

Dwuma-Badu, D., Ayim, J.S.K., Mingle, C.A., Tackie, A.N., Slatkin, D.J, Knapp, J.E., and Schiff, P.L.J., 1975. Alkaloids of *Cissampelos pareira*. *Phytochemistry.*, 14:2520-2521.

Rojanasonthorn, G.. 1970. The isolation and characterization of bisbenzylisoquinoline alkaloid "tetrandrine" from the roots of *C. pareira*. Thesis-Department of Chemistry, Mahidol University, Thialand, pp. 41.

Phytochemistry 1975,9,157.

Morita, H., Mastumoto, K., Takeya, K., Itokawa, H., and Iitaka, Y., 1993. A Novel Antileukemic Tropoloisoquinoline Alkaloid, Pareirubrine, from *Cissampelos pareira. Chem. Lett.*, 2:339-342.

Morita. H., Mastumoto, K., Takeya, K., Itokawa, H., and Iitaka, Y., 1993. Conformation of tropolone ring in antileukemic tropoloisoquinoline alkaloids. *Pharm. Bull.*, 41:1478-80.

Morita H., Takeya, K., and Itokawa, H., 1995. A novel condensed tropone –isoquinoline alkaloid, pareitropone from *C. pareira. Bioorg. Med. Chem.*, 5:597-598.

Morita, H., Mastumoto, K., Takeya, K., and Itokawa, H., 2002. Azafluoranthene Alkaloids from *Cissampelos pareira*. *Chem. Pharm. Bull.*, 41:1307-1308.

Carabot, R.A., Meléndez, P., Carmona, J., Jimenez, M., Patel, A.V., Crabb, T.A., Blunden, G., Cary, P.D., Croft, S.L., and Costa, M., 2003. Cissampeloflavone, a chalcone-flavone dimer from *Cissampelos pareira*. *Phytochemistry.*, 64: 1421.

Amresh, G., Singh, P.N., and Rao, Ch.V., 2001.Antinociceptive and antiarthritic activity of *Cissampelos pareira* roots. *J. Ethnopharmacol.*, 111:531-536

Amresh, G., Zeashan, H., Singh, P.N., and Rao, Ch.V., 2007. Prostaglandin mediated anti-inflammatory and analgesic activity of *Cissampelos pareira. Acta. Pharma.Sci.*,49: 153-160.

Amresh, G., Reddy, G.D., Singh, P.N., Rao, Ch V., 2007. Evaluation of *Cissampelos pareira root in rats. J. Ethnopharmacol.*, 3:526-531.

Ganguly, M., Borthakur, M., Devi, N., and Mahanta, R.,2007. Antifertility activity of the methanolic leaf extract of *Cissampelos pareira in female albino rats. J. Ethnopharmacol.*, 111:688-91.

Amresh, G., Rao, Ch V., and Singh, P.N., 2007. Antioxidant activity of *Cissampelos pareira on* benzo (a) pyrene-induced mucosal injury in mice. *Nut. Res.*,27:625-32.

Amresh, G., Rao, Ch V., and Singh, P.N., 2007. Evaluation of *Cissampelos pareira* against gastric cancer and enzymes associated with carcinogen metabolism. *Pharm. Biol.*, 45:595-603.

Badilla, B., Chaves, F., Jiménez,S., Rodriguez, G., Poveda, L.J., 2008. Effects of an extract of *Cissampelos pareira* on the hemorrhagic and proteolytic activities from *Bothrops asper venom. Pharmacog. Mag.*, 2008.4:27-31.

43, Amresh, G., Rao, Ch V., and Singh, P.N., 2008. Toxicological screening of traditional medicine Laghupatha (*Cissampelos pareira) in experimental animals. J. Ethnopharmacol.*,116:454-460.

44. Pradhan, S.N., Pandey, K., and Badola, R.P., 1964. A clinical trial of hayatine methiodide as a relaxant in 100 cases. *Brit. J. Anest.*, 36:604-611.

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Newsletters

Food for Space

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Overview

Eating aboard a spacecraft is more than just grabbing some fast-food. Biological, operational, and engineering factors play a part in the types of food that are available in a spacecraft. These factors involve the effect of the food on the astronaut, the structure of the food's container, and how manageable the food and container is, respectively.

The following lists factors that determine good space food.

1. Biological Factors: easily digested, nutritious, palatable, safe
2. Engineering Factors: compact, dehydrated, durable, lightweight
3. Operational Factors: easily disposed, easily prepared, lightweight, long shelf life

By the 1960s, NASA achieved an extraordinary technological feat by sending men into space. Yet one deceptively simple aspect of space travel took several more years to perfect: the food. Today most space food looks a lot like food here on the ground. What started out as tasteless paste squeezed out of a tooth-paste like tube has come a long way from space exploration's early days. Astronauts are even getting treated to gourmet meals designed by celebrity chefs.

When astronauts leave for a mission, they will not be able to refrigerate their food, so anything they take along must be nonperishable. The space food that they bring must also be easy to eat without making a mess, since the astronauts will be outside the gravitational field, and any crumbs from their food could float around and get stuck in equipment or in the astronauts' eyes. Because astronauts will often be in space for weeks or months at a time, they are usually unable to bring fresh food, such as fruit and vegetables, on the shuttle. Many types of space food are dehydrated, or freeze-dried, and sealed. When the astronaut is ready to eat the space food, he can add water to rehydrate the meal so that it will be edible. Though there is no refrigerator on a space shuttle, there is an oven, so astronauts will be able to heat up or bake their space food as necessary.

What is space food?

A typical space menu is made up of a lot of the same items found in homes and restaurants here on Earth. It might include foods such as:

- Beef stroganoff
- Brownies
- Crispy rice cereal
- Chicken stew
- Scrambled eggs
- Pineapple
- Granola bars
- Macaroni and cheese
- Chocolate pudding

The biggest differences between space food and regular food are in the packaging and design. Space food must be carefully contained so it doesn't float around in the low-gravity (microgravity) environment. Even something as simple as a few crumbs can become deadly in low gravity. Loose pieces of food can become lodged in shuttle vents or can waft into an astronaut's nose or mouth and pose a choking or breathing hazard. Liquids can float away as well, so drinks like coffee, orange juice, apple cider and tea are packaged as powders. Astronauts add water to the contained drinks to rehydrate them.

While in space, astronauts generally use flour tortillas for sandwiches, rather than bread. This is because bread can have a lot of crumbs, which will float around in the shuttle. For condiments to go on space food, ketchup, mustard, and mayonnaise are provided in their standard forms, but salt and pepper are provided only in liquid form, because the powdered version would be likely to float away.

As on Earth, space food comes in packages that must be disposed. Astronauts must throw their packages away in a trash compactor inside the space shuttle when they are done with eating. Some packaging actually prevents food from flying away.

The food packaging is designed to be flexible, easier to use, as well as maximize space when stowing or disposing food containers.

Astronauts eat 70% less food than people on Earth. Astronauts eat the same food as people on Earth, but their food is specially preserved to avoid contamination by bacteria.

To combat the problem of microgravity, food is carefully contained and drinks are packaged as dehydrated powders. The astronauts add water to beverages through a special tube before drinking.

Astronauts attach their individual food containers to a food tray with fabric fasteners. The tray itself connects either to the wall or to the astronauts' laps. Astronauts open the food packages with scissors and eat with a knife, fork and spoon.

Astronauts don't use bread as the crumbs may float around. Inhaling these crumbs is dangerous. They also avoid hot foods, as it may burn them if it floats onto them.

Space Food Systems:

Historically, space food systems have evolved as U.S. space programs have developed. The early Mercury program (1961–1963) included food packaged in bite-sized cubes, freeze-dried powders, and semi-liquid foods (such as ham salad) stuffed into aluminum tubes. The Gemini program (1965–1966) continued using bite-sized cubes, which were coated with plain gelatin to reduce crumbs that might clog the air-handling system. Freeze-dried foods were put into a special plastic container to make rehydrating easier.

The Apollo program (1968–1972) was the first to have hot water. This made rehydrating foods easier, and also improved taste and quality. Apollo astronauts were the first crew members to use the *spoon bowl*, a utensil that eliminated having to consume food into the mouth directly from the package.

The quality, taste, and variety of foods improved even more during the Skylab program (1973–1974), the only program to have refrigerators and freezers for storage of fresh foods. The menu contained seventy-two different food items.

The Shuttle program, which began in 1981, includes food prepared on Earth from grocery store shelves. With the help of a dietitian, crew members plan individual three-meal-per-day menus that contain a balanced supply of the nutrients needed for living and working in space. Crew members are allowed to add a few of their own personal favorite foods (which may require special packaging to withstand the rigors of spaceflight). Freeze dried foods are re-hydrated using water that is generated by the Shuttle's fuel cells. Foods are eaten right from the package (on individual food trays), or they may be heated in a convection oven in the Shuttle galley.

Designing food for consumption in space is difficult. Foods must meet a number of criteria to be considered fit for space; first, the food must be physiologically appropriate, specifically, it must be nutritious, easily digestible, and palatable.

Second, the food must be engineered for consumption in a zero gravity environment. As such, the food should be light, well packaged, quick to serve, and easy to clean up (foods that tend to leave crumbs, for example, are ill-suited for space). Finally, foods must require a minimum of energy expenditure throughout their use, i.e., they should store well, open easily, and leave little waste behind.

Types: There are several classifications for food that is sent into space:

- *Beverages (B)* - Various rehydratable drinks.
- *Fresh Foods (FF)* - Foods that spoil quickly that needs to be eaten within the first two days of flight to prevent spoilage.
- *Irradiated (I) Meat* - Beef steak that is sterilized with ionizing radiation to keep the food from spoiling.
- *Intermediate Moisture (IM)* - Foods that have some moisture but not enough to cause immediate spoilage.

- *Natural Form (NF)* - Mostly unprocessed foods such as nuts, cookies and granola bars that are ready to eat.
- *Rehydratable (R) Foods* - Foods that have been dehydrated and allowed to rehydrate in hot water prior to consumption.
- *Thermo-stabilized (T)* - Foods that have been processed with heat to destroy micro-organisms and enzymes that may cause spoilage.

More common staples and condiments do not have a classification and are known simply by the item name:

- *Shelf Stable Tortillas* - Tortillas are unleavened flat breads made with corn or wheat flour, water, and salt. Tortillas are heat treated and specially packaged in an oxygen free nitrogen atmosphere to prevent the growth of mold.
- *Condiments* - Liquid salt solution, oily pepper paste, mayonnaise, ketchup, and mustard.

Analysis: Biological, operational, and engineering factors are simply categories by which space food descriptions are split. Due to the space environment and its limitations, the type of food brought into space must be carefully examined.

The health of the astronauts depends on the biological factors in food design. The food must be safe, nutritious, and palatable. It must also be easy to digest and must not cause gastroenterological or hygiene problems.

The engineering factors deal with the weight of the package and food as well as how compact they are for storage. Long voyages require large amounts of food, which must also survive the temperature, pressure, acceleration, and vibration of flight. Food must be dehydrated to make it lighter, more compact, and less likely to spoil. Vehicle mass (weight) is one of the most critical spacecraft aspects, because as weight increases, the fuel and therefore cost required increases.

The operational factors involve both the food and its packaging. The food must have a long shelf life (over 30 days), and the food and its container must be light in weight for easy use. In addition to difficulty in moving things in microgravity, the astronauts may have work to do. Therefore, food must be easy to both prepare and dispose in order to save time.

Plantation in Space:

Astronauts today rely on pre-packaged food, which is not very appetizing and would not be ideal for extended space missions. Plants would be a good source of food, and they could supply oxygen and remove carbon dioxide. Plants have successfully been grown in microgravity. Crops of potatoes, soybeans, lettuce, carrots, wheat, and rice may possibly be grown in space, as scientists are selectively breeding them to be smaller in size but high-yielding in order to maximize production of food in limited spaces.